A RESTRICTION ANALYSIS OF *ESCHERICHIA COLI* *TRP* OPERON DNA

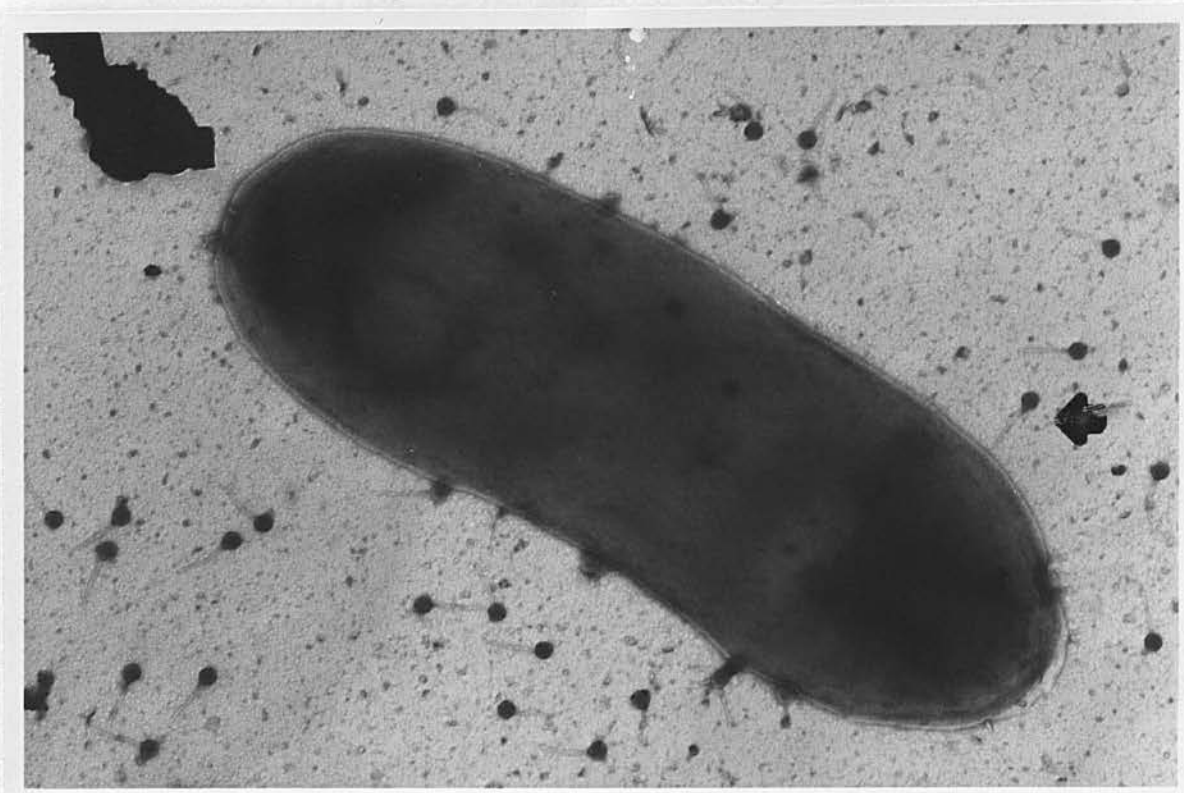
by

Algis Anilionis

A thesis submitted for the Degree of
Doctor of Philosophy of the University
of Edinburgh

Dept Molecular Biology
University of Edinburgh

June 1977



Escherichia coli K12 strain C600 with bacteriophage Lambda (x 60,000)

Glucose grown bacterial cells (with ca. 10-20 lambda receptors per cell surface) were preadsorbed with a concentrated stock of λcI26 in the presence of 10 mM $MgCl_2$. The preparation was stained with 2% Uranyl acetate and picked up directly onto parlodion coated grids and scanned under the electron microscope. The arrow shows an adsorbed phage particle.

CONTENTS

FIGURES, PLATES AND TABLES

CHAPTER 3 (Cont'd)

CHAPTER 4

APPENDIX I

APPENDIX II

Acknowledgements

My sincere thanks go to my supervisor and mentor, Dr W.J. Brammar, for his unfailing encouragement, optimism and patience, and for the many hours of his time he so readily gave in discussion. I consider myself fortunate indeed to have learned from one so able, and one so willing to pass on his expertise and to share his knowledge.

My thanks also to all the people in the department who made my stay so enjoyable, and for me, profitable. In particular, to Dr N.E. Murray, for her forbearance and tolerance, and for access to her unique collection of phage stocks together with advice in their use. To Sue Dunbar for bacterial strains so cheerfully dispensed. To Dr P.J. Highton for instruction in the use of the electron microscope. To Dr John Atkins, Dr Jean Beggs, Graham Brown, Kathy Chalmers, Chris Hadfield, Dr Andrew Hopkins, Steve Hughes, Kim Kaiser, Dr Sue Kingsman, Richard Thompson, Douglas Ward, Dr Geoff Wilson and Dr John Windass for their company and friendship and many fascinating and instructive discussions.

I thank Professor M.R. Pollock and Professor K. Murray for space in their department. Professor Murray supplied sympathetic and well considered advice as well as samples of the more exotic restriction enzymes.

I acknowledge a scholarship from the Medical Research Council for training in research methods.

Finally, I wish to thank my sister Monika Hetherington for skillful and speedy typing of the manuscript.

Foreword

This thesis was composed by me and the work described is my own. The original ideas were conceived and matured by discussion with my supervisor and other members of the department. The experiments were designed and executed by me.

A. Anilionis

ABSTRACT

The genome of a lambda-*trp* transducing phage : λ*trp*$^{+}$51, was used as an enriched source of *E.coli trp* operon DNA. The position in the phage genome of the transduced bacterial DNA and of the *trp* operon within it were determined by electron microscopic analysis of DNA heteroduplexes formed from the *trp*$^{+}$ phage and its λ:Ø80 hybrid parent, 407.5, and also from λ*trp*$^{+}$51 derivatives carrying genetically defined deletions or substitutions. This information facilitated the interpretation of the banding patterns of restriction enzyme digests of λ*trp*$^{+}$51 DNA and those of its derivatives, the λ*trp*51 series, and allowed a restriction map of the *trp* operon DNA to be built up.

The positions of the HindII, HindIII and HpaI recognition sequences within the operon have been determined. The endonucleases AvaI, XhoI and BamHI do not cleave *trp* DNA and their targets flanking the operon in λ*trp*$^{+}$51 have been mapped.

The crossover between lambda and Ø80 DNA in the hybrid progenitor of λ*trp*$^{+}$51 has been located within a 200 base pair region at 65.2-65.6% on the standard lambda map, in an area of partial sequence homology between the two genomes, and places the single site recognised by the *E.coli* K-restriction enzyme in the Ø80 DNA within the 500 base pair region to the left of this crossover.

Alignment of the genetic map of the *trp* operon with the physical map obtained by restriction analysis shows close agreement between the two except in one small internal segment. This finding prompted a search for recombination hotspots in the *trp* operon DNA

and two sites, which may be *chi* (crossover hotspot instigator) sites, have been located within the DNA removed by the *trp*D1 deletion.

A similar survey of *chi*-like sites carried by *in vitro* and *in vivo* recombinant lambda transducing phages revealed seven such sites in the *E.coli* DNA scanned. Extrapolation of these results leads to an estimated total of 250 *chi* sites within the *E.coli* genome.

Lambda NN^- mutants propagate as plasmids, and the Lac^+ and Amp^R phenotypes conferred by λ*lac*NN^- and λ*Tn*ANN^- have been used to select plasmid-bearing clones after infection from a phage particle and after transformation by purified phage DNA. Derivatives of each of these λNN^- phages retaining a single *Eco*RI target have been constructed and their use as molecular vehicles proposed.

NOMENCLATURE & CONVENTIONS

The positions of genetic loci on the linkage map of *Escherichia coli* are taken from Bachmann *et al.* (1977).

The acronymic system of Smith & Nathans (1973) is adopted for the sequence-specific 'restriction' endonucleases. Thus, the restriction enzyme of *E.coli* specified by the R plasmid aggregate of Yoshimori, RY5, is *Eco*RI. The restriction enzymes of *Haemophilus influenzae* serotype Rd are *Hind*II and *Hind*III, and those of *Haemophilus parainfluenzae* are *Hpa*I and *Hpa* II.

The molecular weight of intact λ^+DNA previously estimated as 30.8 million daltons (Md) (Davidson & Szybalski, 1971), has recently been redetermined (Philippsen & Davis, 1976): λ^+DNA is now taken as 49.0 Kilobase-pairs (Kb) i.e. 32.5 Md. This new estimate is used throughout this thesis. Whilst size estimates expressed as % λ^+ length remain unaffected, absolute lengths quoted in Kb or Mdaltons are increased by 5.4%. Any comparison with previously obtained data must take this factor into account.

CHAPTER 1

INTRODUCTION

The trp operon of E.coli

The five genes, specifying the enzyme activities responsible for the biosynthesis of L-tryptophan from non-aromatic precursors are clustered in the genome of the enteric bacterium Escherichia coli, and are subject to co-ordinate control. This gene cluster : the trp operon, and the genetic and biochemical basis of its control is now one of the best understood biological systems of regulation at the molecular level. Much of the early work on tryptophan biosynthesis was with the trp operon of Salmonella typhimurium and subsequent characterisation of the E.coli trp operon showed parallel structural organisation and control in the two systems. Our knowledge of the organisation and control of tryptophan biosynthesis in other organisms, however, is still primitive (See Margolin, 1974, for a detailed review). The trp operon lies at 27 mins. on the standard 0 - 100 mins. circular linkage map of the E.coli genome (Bachmann et al., 1977) and its single transcript specified the trp enzymes in the order of their reaction sequence in the biochemical pathway : Figure 1-1.

Under conditions of tryptophan starvation the operon is transcribed by RNA-polymerase, which binds at the major promoter, trp p_1 and transcribes the entire operon in a 7 Kb messenger (mRNA) molecule (Imamoto et al., 1965; Mosteller & Yanofsky, 1970). Transcription control was demonstrated to be mediated through the interaction of the operator, trpo with the protein product of the trpR gene. This apo-repressor binds the trp operator very

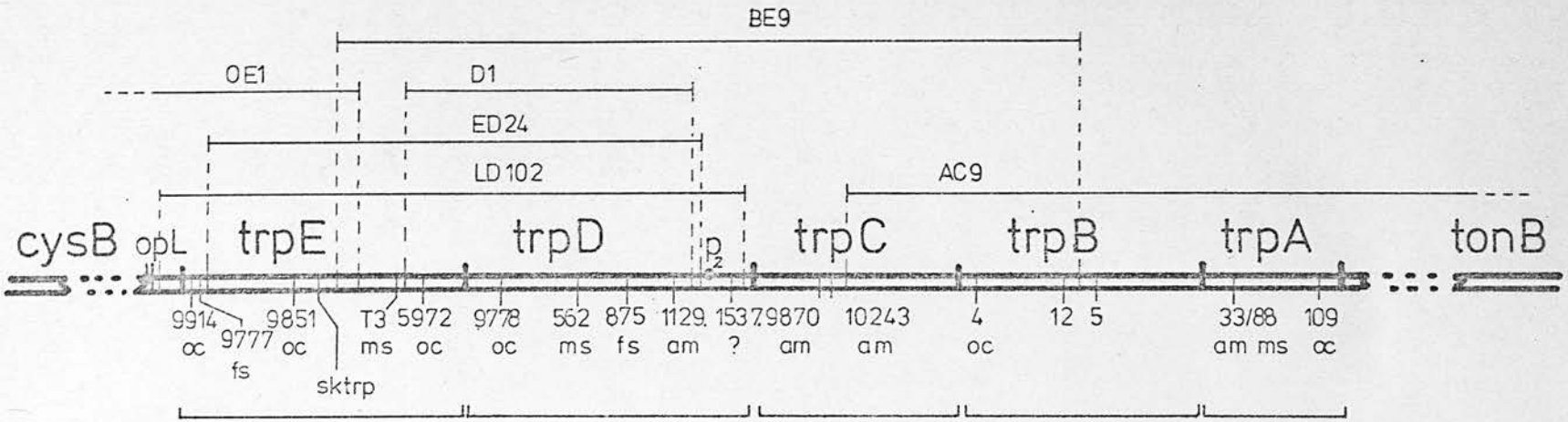
Figure 1-1 The tryptophan biosynthetic operon of Escherichia coli

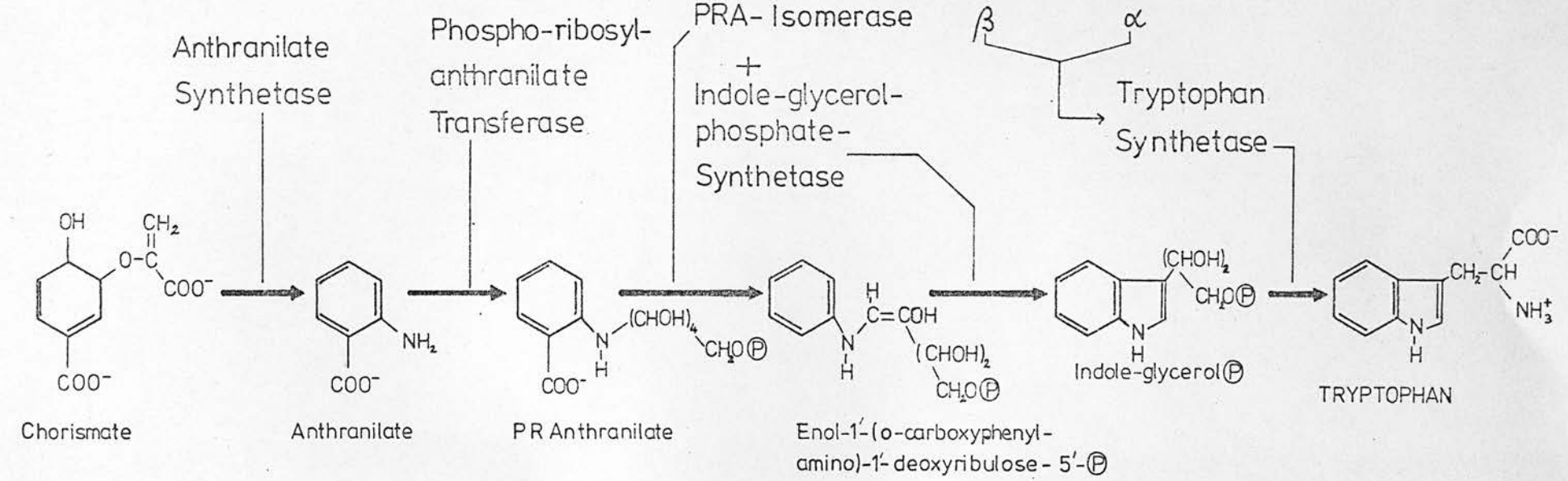
The genetic map of the trp operon and corresponding enzyme activities are shown.

The positions of the trp deletions are taken from Yanofsky et al. (1971); Murray & Brammar (1973) and Jackson & Yanofsky (1972b); The extent of the trpD1 deletion was determined by Anilionis & Brammar (unpublished experiments). The point mutations have been mapped by Yanofsky et al. (1971) and Crawford et al. (1970). The position of the low-level constitutive internal promoter P_2 is taken from Jackson & Yanofsky (1972a).

The trpB product alone is also capable of catalysing the condensation of serine and indole to produce tryptophan, though this activity is stimulated in the presence of the trpA product.

FIGURE 1-1 The E.coli trp operon





tenaciously (K_D = 2 x 10^{-10} M in 0.12M NaCl at 37°C) in the presence of the co-repressor, tryptophan itself (Rose & Yanofsky, 1974) and blocks the binding of RNA-polymerase at trp p_1 and hence prevents new initiations of transcription (Squires et al., 1975). Variation in the concentration of tryptophan in the cell allows a fifty-fold regulation in the rate of synthesis of the trp enzymes by this means.

There is, however, another site of initiation of transcription trp p_2, an internal unregulated promoter within the trp D gene, which allows a low level of expression (a few percent of the maximal) of the trp C,B and A genes during conditions of tryptophan excess (Jackson & Yanofsky, 1972a). This minor constitutive promoter is conserved in several species of enterobacteriaceae (Largin & Belser, 1973).

Examination of the expression of trp enzymes of strains carrying deletions within the operon leaving the trp p_1o region intact (as judged by normal repression by tryptophan) have identified a site between the operator and the first structural gene which may have a regulatory function (Jackson & Yanofsky, 1973). Deletion of this 'attenuator' site results in an eight to ten-fold increase in the rate of synthesis of trp enzymes. These results parallel the finding that there is an approximately ten-fold excess of a small discrete in vivo RNA transcript of some 130 base pairs in length, which is identical in sequence to the 5' segment of the normal trp operon mRNA which precedes the AUG codon for the initiation of translation of the trpE gene (Lee et al., 1976; Bertrand et al., 1976a,b; Pouwels &

Pannekoek, 1976). The region between the site of initiation of transcription and the first structural gene is the trp 'leader' and its 163-nucleotide sequence has been established (Bronson et al., 1973; Bertrand et al.,1975). The foreshortened or 'attenuated' transcript of this region is 144 nucleotides long and is terminated by $-U_7OH$ or $-U_8OH$ and often an uncoded $3'A_{OH}$, in common with several other in vivo transcripts which are known to end in stretches of poly-U, including the E.coli band IV RNA (Ikemura & Dahlberg, 1973), a Ø80 : M3 transcript (Pieczenik et al., 1972) and the lambda 4S and 6S transcripts (Lebowitz et al., 1971).

The trp leader sequence surrounding the attenuator site bears two axes of two-fold rotational symmetry:

DNA: 5'...CGCCCGCGGGCTTTTTTTTGAACAAAAT...-3'
3'...GCGGGCGCCCGAAAAAAAAACTTGTTTTA...-5'

attenuated RNA: 5'...CGCCCGCGGGCUUUUUUUU$_{OH}$

By analogy with other sites of protein : nucleic acid interaction, notably the promoter and operator of the lac operon (Gilbert & Maxam, 1973; Dickson et al., 1975) and the major control complexes o_Lp_L and p_Ro_R of bacteriophage lambda (Maniatis et al., 1974, 1975a,b; Pirrotta, 1975), these may represent recognition sites for specific proteins; in this case those which mediate attenuation. In fact, of those deletions beginning in the leader sequence and running across one of the promoter-proximal structural genes, those which remove the trp attenuator site and its symmetrical sequences also show elevated expression of the remaining trp genes, whilst those which do not suffer altered

regulation leave this site intact (Jackson & Yanofsky, 1973; Bertrand et al., 1975).

There are no apparent accessory requirements for protein factors to achieve attenuation of the trp transcript in vitro (Bertrand et al., 1975), which seems to discount any possibility of dependence on the E.coli termination factor rho (Roberts, 1969). By contrast, there is genetic evidence that expression of the trp operon enzymes is increased two to five-fold by certain ilv-linked suppressor mutations (Korn & Yanofsky, 1976a,b) which also relieve polarity caused by nonsense mutations in the trp operon (Morse, 1972). The situation is confusing in that several phenotypes are observed among the ilv-linked suppressors, some overcoming attenuation to a greater or lesser degree and others including the original suA mutations (which have been shown to directly affect rho : Ratner, 1969) have no effect on attenuation of the trp operon transcript. Thus, in vitro biochemical studies favour an interprelation of trp attenuation involving positive control and relief of termination, whilst the in vivo genetic experiments indicate the involvement of at least one termination factor and negative control of attenuation. It may be that both types of control apply in a complex system of regulation of attenuation in the trp operon.

Attenuation as a mechanism of regulation has also been invoked in the his operon of Salmonella typhimurium (Kasai, 1974), and certain polarity suppressors increase expression of the ilv operon (Wasmuth & Umbarger, 1973) suggesting that attenuation may also be involved in regulation of this operon.

There is an AUG codon in the trp leader mRNA, which if used to initiate translation would lead to the production of a 13-residue peptide in which trp occurs twice, and it has been suggested that this may have some regulatory function; its translation in vivo seems unlikely since there is no G-rich site - postulated as ribosome binding sites distinguishing translation initiation sites (Shine & Dalgarno, 1975) - immediately preceding the AUG codon, but no direct evidence is yet available.

The trp operator and promoter sequences have been deduced from the sequence of 'read-through' transcripts initiated at a Ø80ptrp phage promoter (Bennett et al., 1976). The operator region precedes the transcribed portion of the operon, since one trp deletion mutant LC145-2, which has lost all but the 5'-terminal A residue of the leader sequence retains normal operator function. Two regions of two-fold symmetry are found within this region centred at positions 7-8 and 11-12 base-pairs prior to the initiation site of trp mRNA synthesis and the hyphenated, or partial symmetry extends over a 26 base-pair region which is protected from nuclease attack by RNA polymerase. Purified preparations of trp repressor also protect the trp operator from cleavage by the sequence-specific endonuclease HpaI, as does pre-bound RNA polymerase, showing that the operator and promoter contain common sequences. The trp operon control sequence shows few similarities with sequences characteristic of other promoters in the region 5-12 base pairs upstream of the transcription startpoint (Maizels, 1973; Pribnow, 1975).

Another sequence of interest is that of the trpA-trpB intercistronic boundary (PLatt et al., 1975) which contains the sequence : ....UGAUG.... the UGA codon constituting a 'stop' signal for translation of the trpB polypeptide, whilst the partially overlapping AUG is the initiator codon for the synthesis of the trpA product, and so only two bases between the two genes are not translated.

The trp operon mRNA molecule must contain a transcript of a small region downstream of the last gene in the operon since several trpA frameshift mutations have been shown to produce elongated mutant tryptophan synthetase α-subunit polypeptides (Hardman et al., 1975). The largest of these has a molecular weight of 37.5K, corresponding to an extra 50-70 amino acid residues which must have been translated from an extension of the trp mRNA at least 150-200 nucleotides beyond the trpA gene. Also, several fusions of the lac and trp operons have been isolated which place the lac genes under trp operon control, whilst retaining an intact trpA gene (Mitchell et al., 1976). Thus, these fusions must occur in the untranslated region of the trp operon between the last translation termination site and the transcription termination site, and the total time required for translation of trp messenger in vivo in synchronously derepressed cultures shows that this untranslated region cannot be more than 500 nucleotides in length.

Control of tryptophan biosynthesis in E.coli is also mediated directly by feedback inhibition of tryptophan on the enzyme complex

formed from the products of the trpD & E genes: anthranilate synthetase - PRA transferase (Ito & Yanofsky, 1969); (Henderson et al., 1970). This control follows the classical pattern of feedback inhibition of the end product on the enzymes catalysing the first reaction of a branched pathway, in this case that of aromatic amino acid biosynthesis.

High levels of anthranilate are also known to inhibit the enzymes of the tryptophan synthetase complex (Yanofsky et al., 1971) and though this is probably of little biological significance, it is very useful in the laboratory as a selective pressure for the expression of high levels of the trp operon enzymes.

The bacteriophage Lambda

Lambda is one of a class of temperate bacterial viruses (bacteriophages or more simply, phages) which propagates in E.coli. As a temperate phage propagation is by one of two alternative modes : the lytic mode, characteristic of virulent phages in which infection ultimately results in death of the host followed by cell lysis and release of about a hundred progeny phage particles; the second mode, which is unique to the temperate phages, is establishment of lysogeny. This is a process in which the phage genome integrates into that of the host and is replicated as part of it, being inherited as a genetic trait by each succeeding cell generation. The integrated phage is referred to as a prophage and the host cell which carries it is a lysogen, and exhibits immunity to superinfection by phages of the same type due to the production of an immunity substance : the repressor protein product of the

lambda cI gene. The prophage infrequently enters the lytic cycle spontaneously (10^{-4} per cell generation), but can be quantitatively induced by various treatments including ultraviolet-irradiation (a characteristic of lambdoid prophages, mediated through inactivation of the phage repressor by proteolytic cleavage : Roberts & Roberts, 1975), and alteration of host DNA metabolism by thymine-starvation or addition of mitomycin C.

Lambda (λ),21,Ø80,Ø81,82,424 and 434 all belong to the lambdoid class of phages and they share the properties of being temperate coliphages whose lysogens are inducible by ultraviolet light; they are able to recombine interspecifically and have similar cohesive end sequences at the 5' termini of their DNA molecules. Much of the classic work with Lambda is collected in 'The bacteriophage Lambda' (ed.) Hershey (1971), which provides an excellent source book. Several recent reviews of the physiology of lambda are available, of which those by Thomas (1971), Echols (1972) and Herskowitz (1973) are particularly informative.

The lambda virion consists of a single double stranded DNA molecule 49,000 base pairs in length amounting to a molecular weight of 32.5 million daltons, encapsidated in an icosahedral protein head of a similar molecular weight 500 Å in diameter, from which protrudes a tubular protein tail 1500 Å long. The lambda genome (Figures 1-2 and 1-3) encodes some 35-40 genes which show a remarkable clustering according to function. Seven cistrons necessary for the morphogenesis of the phage head and eleven for the tail have been genetically identified and are specified by the conventional right arm of the linear phage genome. The protein

Figure 1-2 Comparison of Ø80 DNA with that of Lambda

The vegetative genomes of the two phages are aligned so that the major blocks of homology in the left arm of each coincide. (After Fiandt et al., 1971). Strong homology is shown by solid blocks, partial homology by shaded blocks and weak homology by the open rectangles.

The positions of the EcoRI targets in lambda DNA are taken from Thomas & Davis (1975); those in Ø80 DNA are from Helling et al., (1974); The EcoRK targets are from Murray et al., (1973), and the AvaI targets in lambda DNA are from Hughes (1977).

The position of the b522 deletion is taken from Parkinson & Davis (1971) and is relevant to the mapping of the sk80, as described in the text.

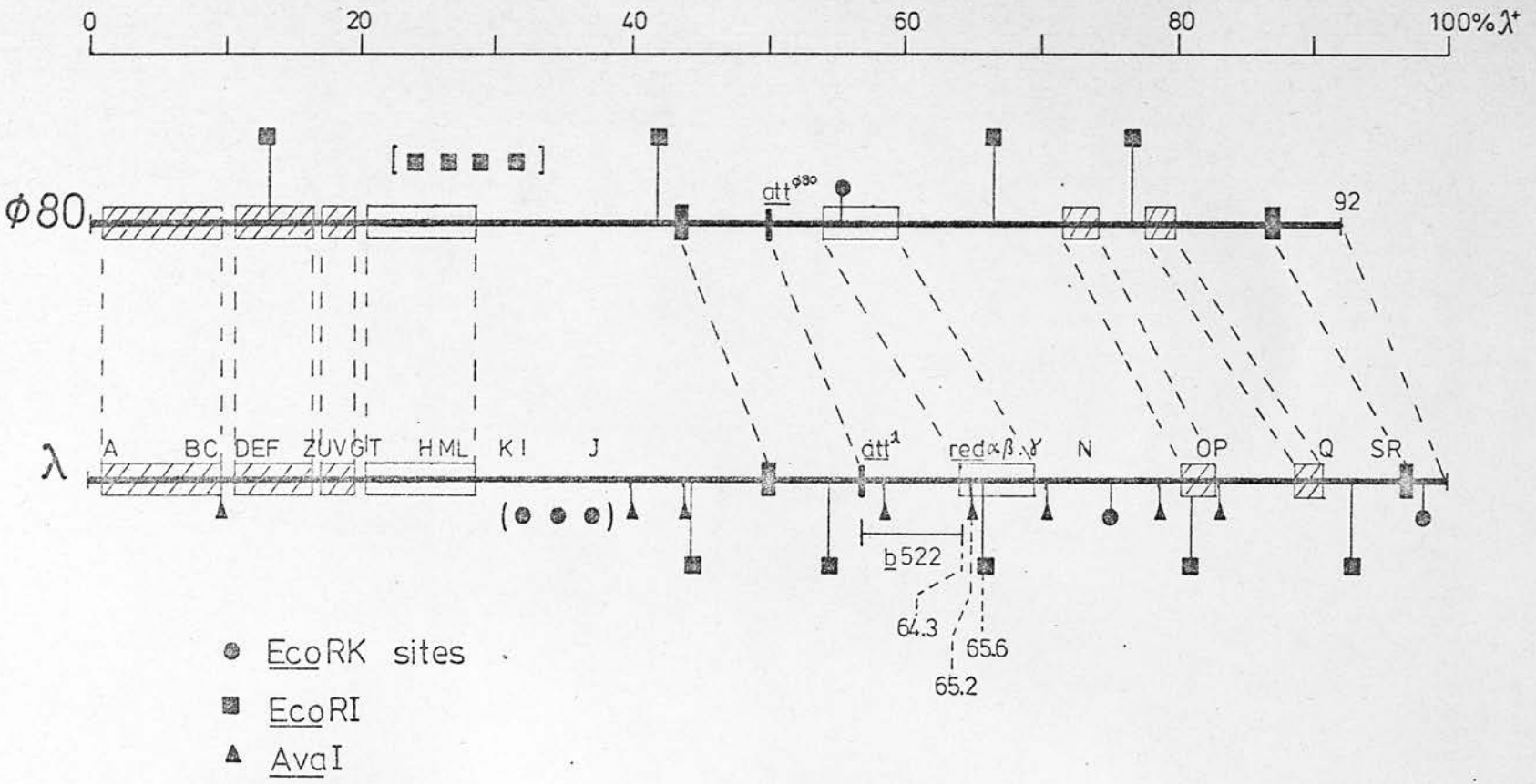


FIGURE 1-2 The genomes of φ80 and λ compared

Figure 1-3 The genome of bacteriophage lambda

Positions of the major genes are indicated by their genetic designations. The map shows the linear vegetative form as packaged in the phage head; clustering of functionally related genes is apparent. Dispensible regions of the genome are shown by shading and the extent of lambda DNA replaced by the immunity substitutions from phages 21 and 434 are indicated by the dotted lines.

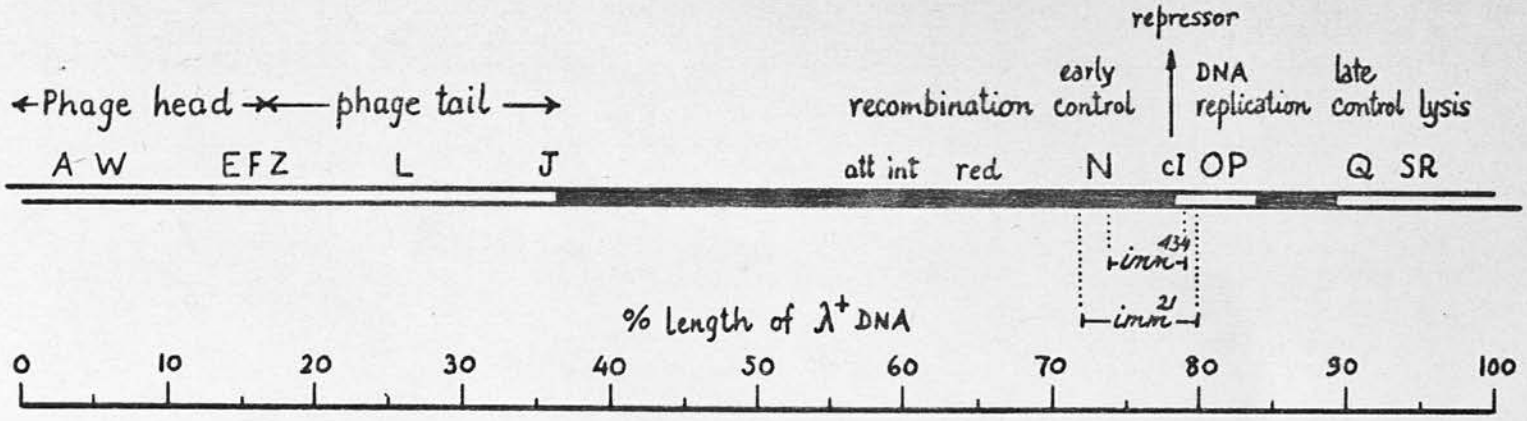
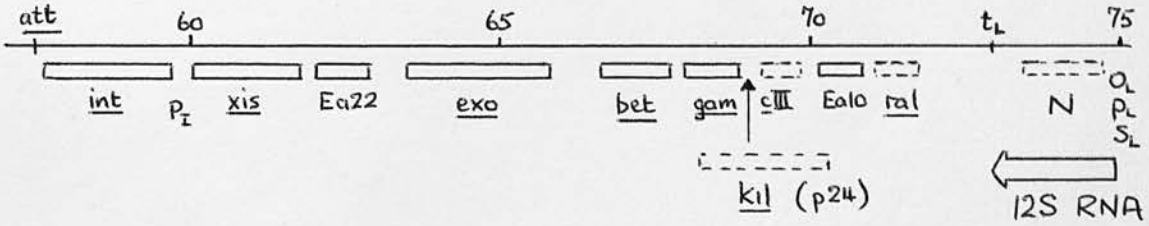

repressor
←Phage head →← phage tail →
recombination
early control
DNA replication
late control
lysis
A W
E F Z
L
J
att int red
N
cI OP
Q SR
imm 434
imm λ
% length of λ+ DNA
0
10
20
30
40
50
60
70
80
90
100

FIGURE 1-3

products of fifteen of these genes have been resolved by gel electrophoresis (Murialdo & Siminovitch, 1972). The central region of about 15% of the genome has not been assigned any function and, though at least three proteins are encoded within it, its deletion does not affect any phage characteristics other than buoyant density and genome length (Parkinson & Davis, 1971). The recombination functions of the major leftward operon are dispensible, but the N gene itself is essential for lytic growth. Determination of the map locations of the N operon genes and the assignment of their sizes deduced from the molecular weights of their protein products show an apparent over-saturation of the coding capacity in the gam-ral segment (Szybalski & Szybalski, 1974):



The positions are expressed as % lambda length from the conventional left end of the linear vegetative molecule. The int and xis functions are required for the site-specific recombination events between the phage attachment site : PP' and the bacterial att : BB'. The int protein alone catalyses the integration of the prophage during lysogen formation and both int and xis proteins are necessary for its subsequent excision. The promoter p_I maintains a low constitutive level of expression of int independently of other control.

The phage also specifies its own recombination functions : redα (or exo), which encodes the lambda exonuclease and redβ (or bet), whose protein product complexes with that of redα and is necessary for its recombinogenic activity (Signer, 1971). The gam protein is an inhibitor of the host exonuclease V to which intermediate forms of the replicating phage are sensitive. The cIII product is required for efficient establishment of repressor synthesis and ral is a protein which has been shown to alleviate the restriction of unmodified phage genomes on infection of a K-restricting host. The lambda Kil function, which is responsible for killing of the host prior to cell lysis during infectious growth, has been shown to be mediated by a protein of molecular weight 24K genetically upstream of cIII by deletion mapping. Ea22 and Ea10 are proteins of 22K and 10K respectively, which have been identified as phage-specific products by electrophoresis of infected cell extracts and assigned locations in the lambda genome by substitution mapping with λbio phages (Hendrix : in Hershey, 1971). The N gene itself is initially expressed from a 12^S early RNA transcript initiated at p_L and terminated at t_L under the influence of the host termination factor rho (Roberts, 1969) and is necessary for the subsequent 'delayed-early' expression of all the genes between t_L and att.

The immunity region contains the cI, repressor gene responsible for the immunity phenotype, and the rex gene which is subject to the same 'immunity' controls as cI and causes exclusion of rII mutants of the virulent phage T4 from lambda lysogens. These two functions are transcribed leftwards, whereas the negative control

gene cro (control of repressor and other things; also called tof : turn-off function; ai : anti-immunity, or fed) and the cII gene (another function necessary for establishment of repressor synthesis) which are also within the immunity region, are transcribed rightwards : Figure 1-4.

Beyond cII the major rightward operon contains the phage replication genes O and P, followed by the positive control gene for late expression : Q. The 'late' genes under Q control are transcribed rightwards from p'_R (Herskowitz & Signer, 1970) and include the lysis functions S, a 'membranase' which shuts off host energy metabolism late in the lytic cycle and R, the phage endolysin, (Dambly et al., 1968) as well as the morphogenetic genes which are contiguous with S and R in the circular replicative phage genome.

On infection of a sensitive host, the linear lambda genome circularises by annealing of the complementary ter sequences of the protruding 5' ends and transcription and replication proceed from a circular genome. Two major transcripts are made at early times after infection both in vivo and in vitro (Roberts, 1969; Blattner & Dahlberg, 1972), a 12S leftward transcript of the N gene from p_L and a 7S rightward transcript of the cro gene from p_R, using host RNA-polymerase and termination factor rho to produce discrete coherent RNA molecules.

The commitment to follow either the lytic or the lysogenic pathway is made very soon after this stage when delayed early functions are expressed in competition with the immunity establishment process (Echols, 1972). The anti-terminator function, N allows the

Figure 1-4 Major transcripts of the lambda genome

The major leftward transcript, complementary to the l-strand, is initiated at p_L and terminated at t_L (the early leftward transcript I) until the N protein is expressed, when termination at this site is overcome and transcription proceeds across the N-operon and beyond the phage attachment site (delayed early leftward transcript II).

The early rightward transcript (I) is initiated at p_R, proceeds across the cro gene and is then inefficiently terminated at t_{R1}, and about one fifth of the molecules are extended across the DNA replication genes O and P and finally terminate at t_{R2}. In the presence of N protein the delayed early transcript (II) is produced so that all functions between cro and Q are expressed at a high rate, and Q in turn stimulates initiation of late transcription (III) which includes the morphogenetic genes A-J downstream of the cos site formed by annealing of the 12 base-pair complementary cohesive end sequences.

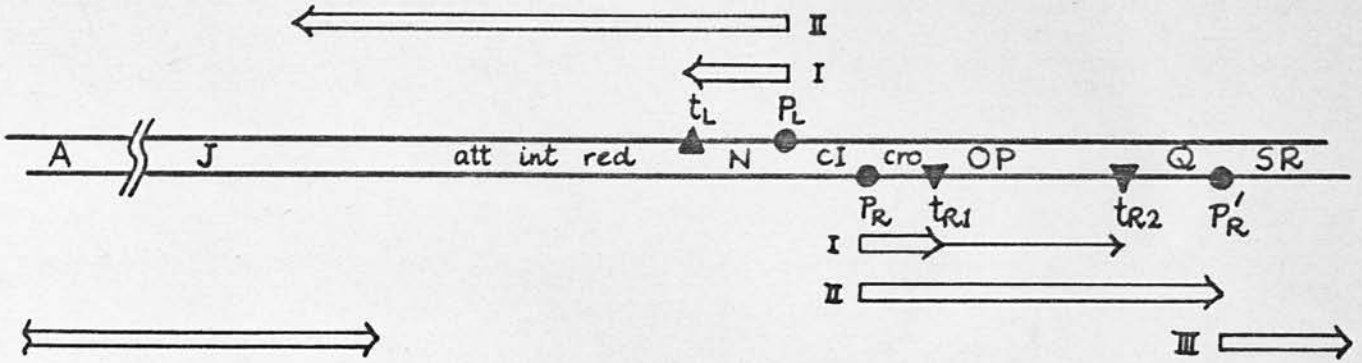
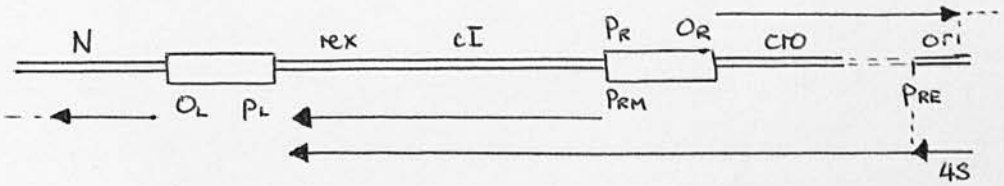


FIGURE 1-4

transcription from p_L and p_R to override their respective terminators t_L and t_{R1} and t_{R2}, and thus allows expression of the N operon and phage replication genes from 2-3 mins. after infection. Concomitantly, the cro protein concentration in the cell builds up until about ten minutes after infection when a steady state is achieved and transcription of its own gene and the delayed early genes is depressed about five-fold by its inhibitory action at the major leftward and rightward control complexes $o_L p_L$ and $p_R o_R$.

The metabolic state of the infected cell probably influences the competition between the positive control gene N, which favours activation of the late genes by stimulation of Q synthesis and consequent propagation in the lytic mode, and the repressive activity of cro product on this activity at the major rightward control complex $p_R o_R$. The latter interaction favours establishment of repressor synthesis, which requires cII and cIII proteins acting in concert at p_{RE}, specifically stimulating the extension of the 4S RNA transcript from ori. This small oop RNA appears to serve a dual function as primer for DNA replication initiation and also an attenuated mRNA (Honigman et al., 1976) whose extension produces an anti-sense transcript of the cro gene and proceeds across $p_R o_R$ and finally transcribes the cI and rex genes. Once repressor protein is synthesised it efficiently blocks all transcription from p_L and p_R by specific tight binding at o_L and o_R, and shuts off Q expression. Thus competition between the two modes of transcription across the cro gene in the alternative senses may be the ultimate arbiter of the decision between lysis and lysogeny within 15-20 mins. after infection.

The organisation of cI transcription and its regulatory sites are detailed below.

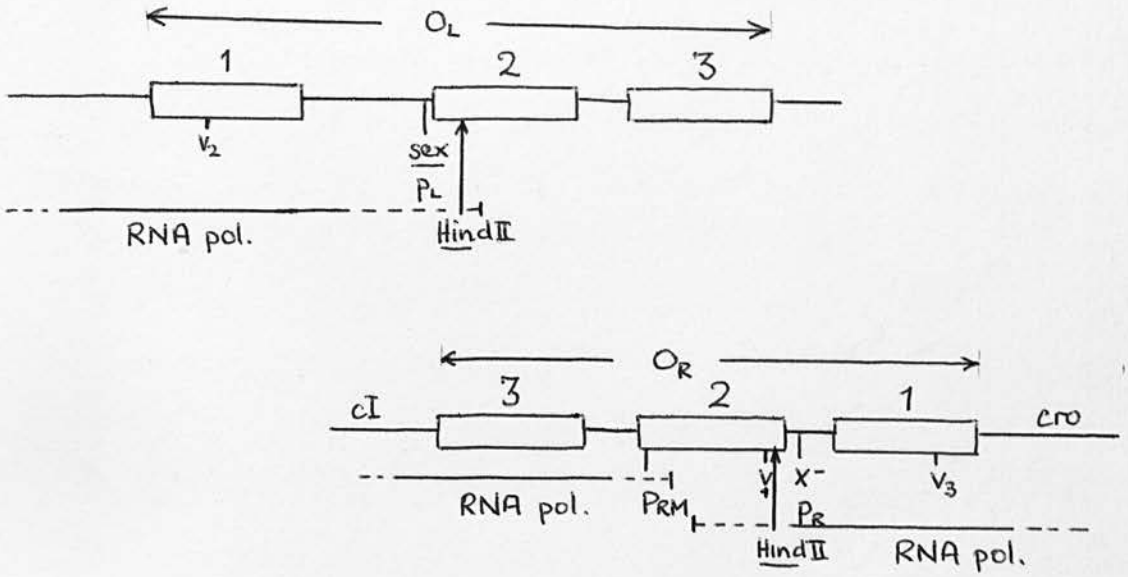


λ Repressor, an acidic protein whose MW has been variously measured as 26K and 28K, acts at the two major operators o_L and o_R and from these two sites mediates an impressive array of control functions: Binding at the two major operators directly inhibits transcription from p_L and p_R, and its action at these two sites suffices to turn off the expression of most phage genes. Secondly, transcription from p_{RE} for establishment of repressor synthesis is blocked at o_R by the binding of repressor itself and since repressor activity also prevents further cII and cIII expression, transcription from p_{RE} is quickly halted. Thirdly, repressor stimulates its own synthesis when present in low concentrations in the cell by effecting shut-off of transcription from p_R and favouring transcription from the other strand at the neighbouring promoter p_{RM} which can only compete for RNA-polymerase binding with the help of bound repressor. Continued cI expression from p_{RM} elevates its concentration in the cell until a level is reached where binding of more repressor at o_R blocks this expression too, thus constituting a finely regulated autogenous control of repressor synthesis. Finally, since replication of the phage DNA requires ongoing rightward transcription across the ori site (Dove et al., 1971) repressor also exerts an epistatic control over initiation of phage replication.

Since the nature of the operator : repressor interactions in these controls serves as an illustration of the use of restriction analysis and DNA sequencing, these will now be considered in more detail.

Both major lambda operators contain three repressor dimer binding sites, each 17 base pairs long and separated by AT-rich spacer regions of 3-7 base pairs. The site which is initially bound in each operator is that nearest the operon it controls and adjacent sites are subsequently bound in order. Regions of about 25, 50 and 80 base pairs in length are protected from digestion by non-specific nucleases *in vitro* by increasing concentrations of repressor (Ptashne *et al.*, 1976; Maniatis *et al.*, 1974 & 1975) and these correspond to the binding sites 1; 1 & 2 and 1, 2 & 3 in each operator.

The sequence-specific restriction endonuclease HindII cleaves each operator once and the nucleotide sequence of the operators have been determined from the four fragments produced by HindII in this region and the specific binding sites within them determined by studies of protection from methylation of A and G residues of their DNA *in vitro* by pre-bound repressor. These results suggest that lambda repressor specifically interacts with the minor groove of its DNA binding site. Determination of sequence changes due to virulence mutations (conferring insensitivity to repressor) and various promoter defects have allowed assignment of functions to segments of the major control complexes and show interpenetration of the operators and promoters:



The sites of action of cro protein are also within these sequences since the vir mutations of the operators have been shown to decrease its effectiveness in inhibiting transcription from p_L (Sly et al., in Hershey, 1971) and p_R (Berg, 1974).

The switch from p_R to p_{RM} promoted transcription is effected by repressor binding at o_R1 channeling RNA polymerase into the p_{RM} site, and subsequent binding of repressor at o_R2 & 3 blocks transcription from both (Pirrotta, 1976). Only about 200 repressor monomers are present in an ordinary lysogen maintained by p_{RM}, and translation of this mRNA is only one-fifth as efficient as that of the p_{RE} transcript, an effect consistent with the absence of a leader sequence in the former and the consequent loss of the ribosome binding site (Shine & Dalgarno, 1975) prior to the AUG codon for initiation of translation of repressor protein (Ptashne et al., 1976).

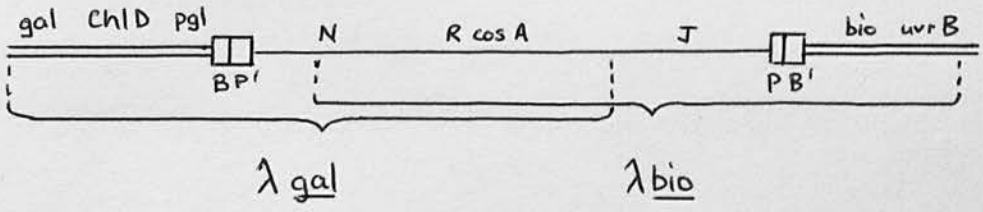
Equilibrium dialysis studies show that lambda repressor specifically binds the operators of the lambda immunity region and not that of λimm434 and has a much lower affinity for single

stranded or other double stranded DNA. Equilibrium binding follows a sigmoid curve at low repressor concentrations titrated with increasing amounts of lambda DNA (Pirotta, 1976). Binding of the active repressor species - presumed to be a dimer, but the data could also apply to a tetramer - is rapid, the forward reaction rate constant, $K_f = 3.10^{10} M^{-1} l.sec^{-1}$; unless the repressor is first diluted, when recovery of binding is slow but the same equilibrium level is reached. The repressor multimer dissociation constant, $K_1 = [R]^2 / [R_2] = 7.10^{-9} Ml^{-1}$. whereas the repressor : operator dissociation constant,

$$K_D = [R_2] \quad [O] \; / \; [R_2O] \quad = 3.10^{-14} Ml^{-1}.$$

Integration of the prophage during lysogenisation is mediated by the int protein alone, catalysing the reciprocal site-specific recombination event between the two circular DNA molecules, the phage and host genomes, forming a single circular chromosome with the integrated prophage (Campbell, 1962). The int protein also mediates site specific recombination between two phage genomes (Weil & Sigmer, 1968). Both the int and xis functions are necessary for excision of the prophage by the reverse process.

Abnormal excision events (about 1 in 10^5), also int and xis dependent, due to crossovers at sites other than P.P' and B.B' lead to formation of transducing phages carrying small portions of the E.coli genome adjacent to the prophage:



The abnormal excision of a Ø80 prophage, which integrates near *trp* in the host chromosome, leads to Ø80p*trp* transducing phages by a mechanism parallel to λp*bio* formation, and to Ø80d*sup*F in an analogous way to λd*gal* formation.

Lambda will integrate at different chromosomal locations when the normal bacterial attachment site is deleted (Shimada, 1972) again by an *int* catalysed process, at a frequency 200-fold lower than lysogenisation of the wild type host. A further 200-fold reduction is observed in the integration of λ*int*⁻ in such *att*-deletion hosts. Subsequent excision of these unusual prophages has led to the generation of lambda-transducing phages carrying several host genes which are unlinked to the lambda attachment site.

The replication of lambda DNA will now be discussed in detail as it is of special significance to the work to be described.

Replication of lambda DNA

On infection of a normal sensitive host the linear DNA molecules injected by the phage are rapidly converted to covalently closed circles (Young & Sinsheimer, 1964), resulting in the loss of infectivity of re-extracted lambda DNA (Dove & Weigle, 1965). Replication of these circles has been shown to proceed through two phases : First, the early mode in which the monomeric circular phage DNA is replicated bidirectionally (Schnös & Inman, 1970) and semiconservatively from a unique *ori* site (Dove *et al.*, 1971)

producing 'θ' form intermediates and yielding about twenty monomeric circles fifteen minutes after infection (Carter & Smith, 1970). Many of these circles have an interruption in at least one of their strands (Young & Sinsheimer, 1968). Second, the late mode which is characterised by the accumulation of concatenated forms and proceeds from 'σ' shaped intermediates. This mode continues until 50-60 minutes after infection, when cell lysis is achieved and about 80% of the lambda DNA in the cell has been packaged into phage heads (Enquist & Skalka, 1973).

The early mode has been shown to require the function of lambda O and P proteins acting in concert with host replication machinery (Georgopoulos & Herskowitz, 1971; Sunshine et al., 1977). A small, 4S RNA molecule complementary to the l-strand of the ori region, the oop RNA, is thought to serve as a primer for DNA synthesis (Hayes & Szybalski, 1973) displacing the opposite strand (where initiation of replication must also occur); the two newly formed replication forks proceed in opposite directions until they collide at a position diametrically opposite ori (Valenzuela et al., 1976) and the two circles separate.

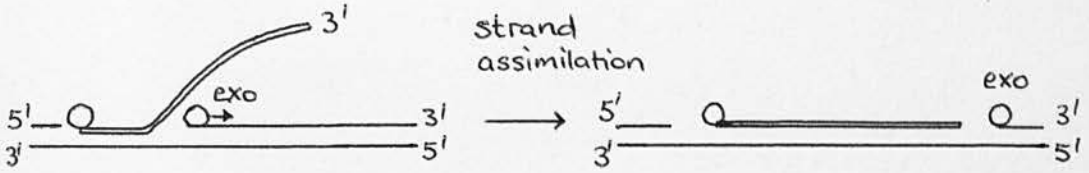
The late mode of replication is marked by the appearance of linear DNA tails protruding from the monomeric circles, these being continuously extended, resulting in the accumulation of multimeric linear concatenates, in a process which is still dependent on functional lambda P product (Takahashi, 1977). Net synthesis of closed circles ceases, though some multimeric circular forms can be detected (Enquist & Skalka, 1973). Gilbert & Dressler (1968)

proposed a model to account for the formation of long concatenated intermediates observed during the replication of a variety of phages. According to their 'rolling circle' model the unbroken circular strand is a template from which concatenated linear copies can be continuously replicated. The predictions of this model have been vindicated for lambda in the late mode of replication (Takahashi,1975 & 1977).

Monomeric lambda DNA, whether linear or circular, has been shown to be a very poor substrate for the maturation and packaging system of lambda (Hohn, 1975 a & b; Hohn & Hohn, 1974), whereas linear or circular multimeric forms are efficiently matured and encapsidated (Stahl et al., 1972). In fact, it has been shown that maturation requires two or more cohesive end (cos) sites per DNA molecule (Freifelder et al., 1974), linear monomers being cut from these multimeric forms by the action of the lambda A gene product (ter) at these sites in a process intimately involved with the morphogenesis system (Wang & Kaiser, 1973).

The phage red and host rec specified recombination systems are both involved in lambda DNA replication and these interactions will now be considered. The 5'-exonuclease activity of the redα gene product is only recombinogenic in the presence of the redβ protein, though the exonuclease activity is not detectably affected by loss of the β protein, (Carter & Radding, 1971 a,b). Lambda exonuclease shows a remarkable specificity and acts only on triple stranded structures in a mode referred to as 'strand assimilation' and though it binds at nicks and free 5'-projections it shows

negligible activity at these sites, leading directly to a possible mechanism of red catalysed recombination. (Cassuto & Radding, 1971.)



Lambda red⁻ mutants, when grown in rec⁺ or recA hosts replicate their DNA at a lower rate and the average size of concatemers at late times is shorter than the wild type. Maturation and packaging of the phage DNA is also less efficient and the phage yield is only 30-40% that of the normal burst. Thus, the red⁻ defect in phage specified recombination also manifests itself in a reduced ability to initiate or to propagate the late mode of replication.

The gam gene produces an inhibitor of the host recBC nuclease (exo V) and its role in phage replication is shown by the reduction in burst size of lambda gam⁻ phages and a further reduction in the extent of late replication in red⁻ mutants. See Figure 1-5.

The effect of red⁻ and gam⁻ mutations on the growth of lambda on various hosts constitute distinct phenotypes:

	Phenotype:	W.T.	FEC	FEB	SPI
	host:	W.T.	recA	pol⁻/lig⁻	(P2) lysogen
Phage :	λ⁺	++	++	+ +	-
	λ red⁻	+	+	-	-
	λ gam⁻	+	+	-	-
	λ red⁻ gam⁻	(+)	-	-	(+)

Figure 1- 5 Lambda DNA synthesis and phage production

Phage DNA synthesis was determined by ^{3}H-Thymidine incorporation into lambda DNA as cpm hybridised to excess cold lambda DNA. Phage production was measured as pfu per singly infected cell.

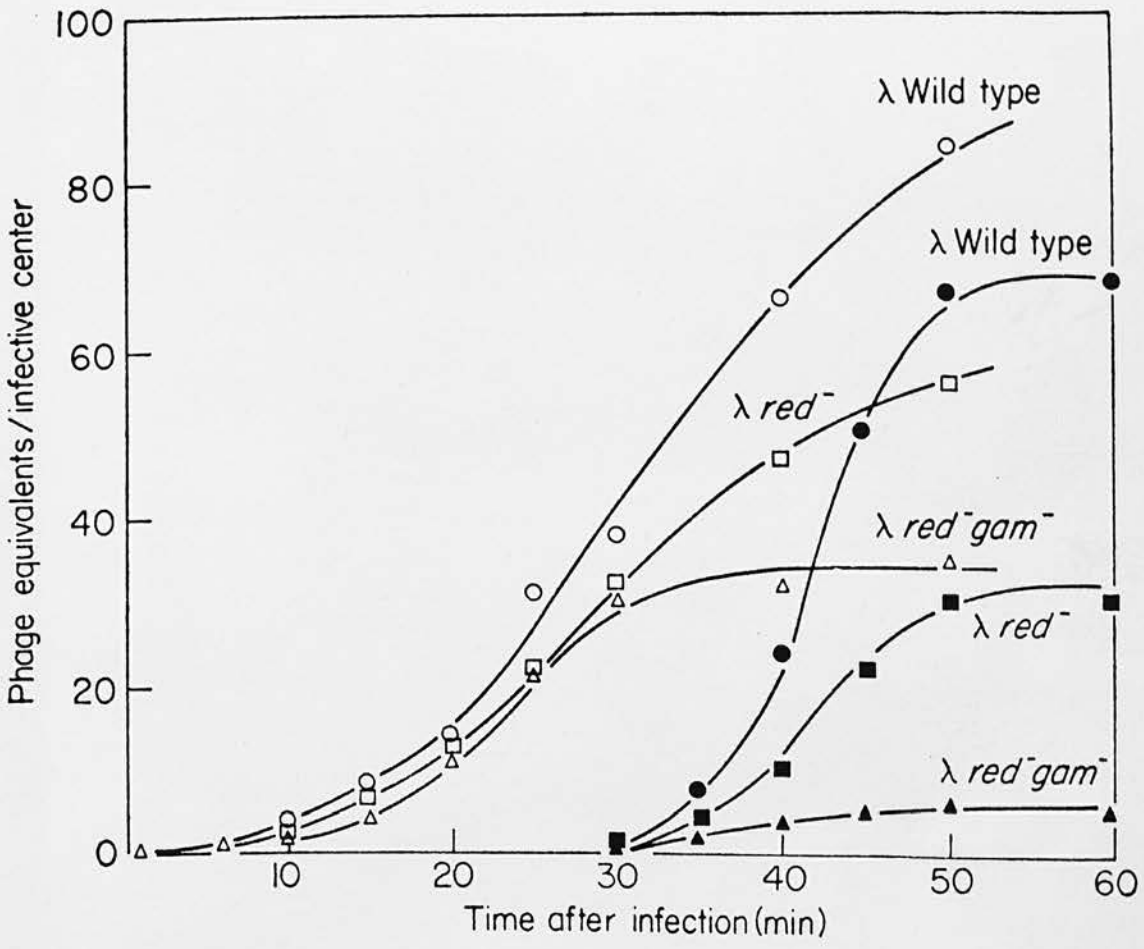


FIGURE 1-5 Lambda DNA synthesis and phage production

(from Enquist & Skalka, 1973)

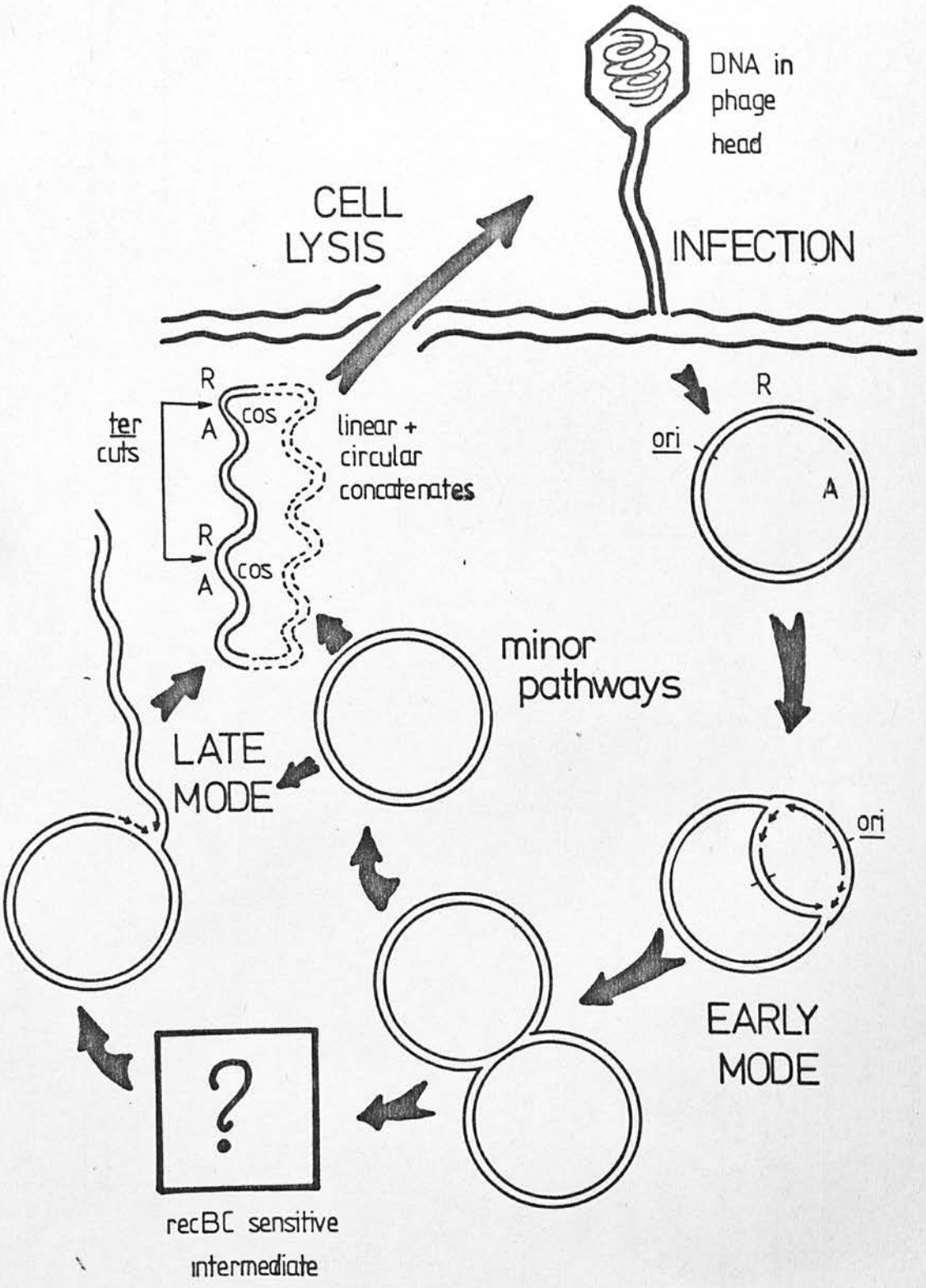

DNA in phage head
CELL LYSIS
INFECTION
R
A
cos
ter cuts
linear + circular concatenates
R
A
cos
R
ori
A
minor pathways
LATE MODE
ori
EARLY MODE
?
recBC sensitive intermediate

FIGURE 1-6

The (+) phenotype is that shown by λ^+ in each case (Zissler et al., 1971 a,b). λ red$^-$gam$^-$ makes tiny plaques even on a wild type host and shows the partial-Spi$^-$ phenotype by poor growth on P2-lysogens unless accompanied by a chi recombination hotspot for the rec system (Malone & Chattoraj, 1975). The Fec$^+$ and Spi$^-$ phenotypes are thus mutually exclusive in unsubstituted lambda, but a transducing phage which has both these properties has been selected. This phage, λ-reverse (Gottesman et al., 1974) carries the sbcA gene of E.coli substituted for the att-red-gam segment of the phage.

A model for lambda DNA replication has been built up from the known characteristics of various lambda mutants and this is presented in Figure 1-6. The transition from early to late modes is mediated by the host rec and/or the phage red systems, and thus probably involved concatemer formation by recombination, but loss of both functions results in poor yields of phages and the few progeny produced arise from maturation of the concatenated forms produced by a 'leakage' into the late mode of DNA replication which is sensitive to attack by the recBC nuclease (Barbour & Clark, 1970) at the transition stage; this is shown by the inviability of λ red$^-$gam$^-$ in recA host, where the recBC enzyme is not inhibited, and the reduced efficiency of rolling-circle formation but not propagation, in replication of λ red$^-$gamts DNA when shifted from the permissive to the non-permissive temperature (Greenstein & Skalka, 1975; Sogo et al., 1976).

Lambda can exist as a plasmid when its replication is locked into the early mode and late functions are not expressed. λ*dv*, a plasmid consisting of a 15% core region of lambda DNA (Matsubara, 1976) which lacks the structural genes for late functions, and λ NN⁻ which lacks the means to switch on their expression, both fulfil these requirements. When λNN⁻ infects a sensitive host the two normal routes to lysis or lysogeny are both blocked by lack of N-dependent anti-termination activity. The simpler situation prevails where only the 12S *m*RNA from p_L specifying inactive N product, and the 7S *m*RNA from p_R specifying active *cro* product are made. Synthesis of *cro* continues until its ability to repress its own transcription from p_R leads to a steady state where *cro*-mediated 'anti-immunity' is established as in the case of λ*dv*'s (Berg, 1974). The 7S *cro* transcript continues to be made to maintain the levels of *cro* activity necessary to retain the steady state and hence establishing an autogenous regulatory circuit; but the 7S transcripts are only inefficiently terminated at t_{R1}, and some 20% continue beyond and transcribe the O and P genes and finally terminate at t_{R2}. Thus O and P functions are expressed by leakage, and since these are the only lambda products required for replication of lambda DNA circles (Ogawa & Tomizawa, 1968) the lambda genome is stably established as a plasmid with some 30-50 copies of the monomer per cell (Lieb, 1970).

λ dv plasmids containing mutant o_R sites which also affect the cro site of action have altered levels of cro expression in the steady state (Berg, 1974), the $v_1 v_3$ mutations increasing the equilibrium concentration of cro in the carrier cell so that λ vir is unable to make a plaque on this host, and this level is in turn exceeded by λ dv-carriers with $v_1 v_3 v_S$ mutations in o_R.

Restriction endonucleases and in vitro recombinant DNA

The phenomenon of host controlled restriction and modification is mediated by enzymes which fall into two distinct groups: those which show a dependence on ATP and SAM (S-Adenosyl-methionine) as well as the cofactor Mg^{++}, and those which have no requirements other than Mg^{++}. The former are the Type I enzymes which have specific recognition sequences but produce random breaks in DNA which contain these sites which are not specifically methylated; the Mg^{++}-dependent enzymes are of Type II which cleave within or near their recognition sequences (See Arber & Linn, 1969; Boyer 1971 and Roberts, 1976 for reviews).

It was quickly realised that the Type II enzymes provide a unique opportunity to produce small coherent DNA fragments from the huge DNA molecules which constitute the genomes of prokaryotic and enkaryotic organisms, and rapid progress was made in the mapping of small segments of specific DNA regions of interest. (Allet, 1973; Allet et al., 1973; 1974; 1975; Maurer et al., 1974.) Also all fragments produced by the action of the same enzyme on DNA from widely different sources have identical end sequences and

so religation in a new fragment order or of DNA molecules from unrelated sources allows *in vitro* DNA recombination across all genetic barriers. Recovery of the DNA and its biological activity in *E.coli* cells has been routinely demonstrated with plasmid λ *dv* (Mukai *et al.*, 1976) lambda (Murray & Murray, 1974; Murray *et al.*, 1977; Borck *et al.*, 1976) as well as other plasmids (So *et al.*, 1975; Collins *et al.*, 1976; Jackson *et al.*, 1972), and expression of eukaryotic genes achieved in *E.coli* by these means, albeit inefficiently (Struhl *et al.*, 1976; Ratzkin *et al.*, 1977). 'Flush-ends' with no 3' or 5' protrusions have also been shown to be accessible to this method (Sgaramella, 1972) and single strand projections added *in vitro* have been used to effect joining of isolated DNA fragments (Jackson *et al.*, 1972). This technique has been used to produce 'clones' of the rabbit β-globin gene in *E.coli* plasmids (Rugeon, 1975; Rabbitts, 1976) and promises to be of immense value in the elucidation of control of eukaryotic gene expression.

Recombination hotspots in lambda and E.coli DNA

Chi (crossover hotspot instigator) sites generated by mutation were first noticed as extragenic suppressors of the small plaque phenotype of lambda phages in which the *red* and *gam* genes were removed by deletion (Henderson & Weil, 1975). These phages were selected by their ability to grow on a P2-lysogen (the Spi^- phenotype : Lindahl *et al.*, 1970), the wild type and the single λ *red*$^-$ and λ *gam*$^-$ mutants being excluded by lysogens of this unrelated phage (the Spi^+ phenotype).

Henderson observed that stocks of these partial Spi$^-$ deletion derivatives were quickly outgrown by phages forming larger plaques which now carried an additional unlinked mutation; the frequency at which these suppressor mutations arose was increased by 2-aminopurine, suggesting that some, at least, were transitions. These suppressor mutations were shown to restore the burst size of λ *red*$^-$ *gam*$^-$ from 20% to 100% that of the wild type, even though lambda specific DNA synthesis was only increased from 35 to 60% wild type levels. The presence of such a suppressor mutation partially restores the level of endolysin synthesis and reduces production of serum blocking protein (the J product : Buchwald & Siminovitch, 1969) to normal. Later Stahl *et al.* (1972) and Lam *et al.* (1974) showed that there were at least four sites in the lambda genome which could be mutated to produce a *chi* site, and McMilin *et al.* (1974) showed that the *E.coli bio* operon DNA contains a naturally occurring *chi* site.

Stahl *et al.* (1975) showed that these *chi* mutations were hotspots for initiation of recombination, even if the site was within a region of non-homology between the interacting molecules and this activity was abolished by *rec*B mutations in the host. This latter finding contrasts with the results of Henderson & Weil (1975) who showed a residual stimulation of recombination in a *rec*B host.

An interesting parallel phenomenon occurs in *Neurospora crassa*, where the wild type *cog* (conversion of genes) allele in linkage group I behaves as a site which strongly competes for

initiation of recombination along the chromosome bearing it, and this activity is also effective when *cog*$^{+}$ is present in a region of non-homology between the paired chromosomes (Catcheside & Angel, 1974). In the absence of the *rec2*$^{+}$ allele, *cog*$^{+}$ allows six times as much allelic recombination in the neighbouring *his-3* gene and nearly four times as much recombination in the *his-3* to *ad-3* interval, and conversion in the strand carrying *cog* is preferred.

CHAPTER 2

MATERIALS & METHODS

1. BACTERIAL STRAINS used in the work described are listed below. Bachmann(1970) provides a useful guide to the ancestry of many of these strains. With the exception of C-1a, all are Escherichia coli K12 derivatives. Lysogens and phage resistant strains were constructed as described in section 5(e) and 5(f), or obtained from the collection of Dr W.J. Brammar.

(WJB) Dr W.J. Brammar (RT) Dr R. Thompson

(NEM) Dr N.E. Murray (GGW) Dr G.G. Wilson

(JGS) Dr J.G. Scaife

Designation	Relevant genotype	Source	Reference
C600	supE, tonA, thr, leu, thi.	NEM	Appleyard (1954)
C600(P2)	P2-lysogen of C600	NEM	
W3350	sup^o, gal, prototroph	WJB	Campbell (1961)
W1485	F^+, supE, prototroph	WJB	Franklin & Dove (1969)
CR63	supD, λ^R,	NEM	Appleyard et al. (1956)
Ymel	supF, cured of λ prophage	NEM	Goldberg & Howe (1969)
594	sup^o, gal, str^R.	NEM	Campbell (1961)
594(P2)	P2-lysogen of 594	NEM	Henderson & Weil (1975)
QR47	supE, prototroph	NEM	Weil & Signer (1968)
QR48	recA1 derivative of QR47	NEM	Weil & Signer (1968)
803	$hsdS^-$, metB	NEM	Wood (1966)

BACTERIAL STRAINS (Cont'd)

Designation	Relevant genotype	Source	Reference
ED8659	*hsdS*$^+$, R$^-$, M$^+$, *trpR*, *supF*, (*tonB*-*trpABC*)∇ AC9	WJB	Borck *et al.* (1976)
5K	*hsdS*, derivative of C600	NEM	Hubacek & Glover (1970)
5K/R1	*str*^{R}, derivative of 5K carrying RI plasmids	NEM	Brammar *et al.* (1974)
KB8	*polA1*, *endA*, *sup*^{o}	NEM	Murray *et al.* (1973a)
N3098	*ligts*^{7}, *supF*	NEM	Pauling & Hamm (1968)
groN785	*groN*, *galE*, *sup*^{o}	WJB	Georgeopoulos (1971)
SA291	(*gal*, *att* , *bio*, *uvrB*)∇ *his*, *str*^{R}, *sup*^{o}	WJB	(Dr S. Adhya)
WB738	*CysB* derivative of SA291	WJB	
C-1a	*E.coli* strain C, *sup*^{o}, prototroph	NEM	Bertani & Bertani (1970)
ED8538	*lac z am*, *sup*^{o}	WJB	
JC5088	*Hfr* (KL16-type), *recA56*, *spc*^{R}	WJB	Clark (1967)
XA21	*lacI3*, *lac z*$^{\nabla}$M15, *thi*, *str*^{R}, *sup*^{o}	JGS	

BACTERIAL STRAINS (Cont'd)

Designation	Relevant genotype	Source	Reference
M5000	glnA3, trpAoc9825, strA176,	NEM	Mayer et al. (1975)
H1SB463	hisB463 (Formerly designated hisC463)	WJB	Struhl et al. (1976)
UTH2743	hisG1	WJB	Goldschmidt et al. (1970)
WB566	trp∇AE8, tna	WJB	
ED8149	trp ∇ BE9, thyA	WJB	Murray & Brammar (1973)
W3110	sup° prototroph	WJB	Yanofsky & Ito (1966)
trp⁻ strains:	All derivatives of W3110		
	trpEam9914	WJB	Yanofsky et al. (1971)
	trpEoc5972	WJB	Yanofsky et al. (1971)
	trpDoc9778 supF	WJB	Yanofsky et al. (1971)
	trpCam9870Cam10243	WJB	Yanofsky et al. (1971)
	trpB4	WJB	Crawford & Johnson (1964)
	trpB9700	WJB	(Dr C. Yanofsky)
	trpAms33, leu am, tonA, supF	WJB	Drapeau et al. (1968)
	trpAam88, trpR, supF	WJB	Drapeau et al. (1968)
	trpAoc109	WJB	Yanofsky et al. (1967)
	(tonB, trpABC)∇ AC9	WJB	Yanofsky et al. (1971)
	trp∇LD102 (formerly trp∇ED102)	WJB	Jackson & Yanofsky (1973)

trp strains (Cont'd)

		Source	Reference
	trp∇ ED24	WJB	Jackson & Yanofsky (1973)
	trp∇ D1	WJB	M.G. Burdon unpublished
	trp ∇BE9	WJB	Murray & Brammar (1973)
	trp ∇OE1	WJB	Murray & Brammar (1973)
	(tonB, trpAE) ∇ AE8, str^R	WJB	Yanofsky et al. (1971)
A125	lacI3lacZ$^{\nabla}$M15, tsx^R (gal, att λ ,bio, uvrB)∇ trp∇LD102, his, sup^o		This thesis
A126	lacI3lacZ$^{\nabla}$M15, tsx^R, (gal, attλ , bio, uvrB)∇ supF, his		This thesis
A129	lacI3lacZ$^{\nabla}$M15, tsx^R, (gal, attλ , bio, uvrB)∇ trp∇LD102, recA56, sup^o		This thesis

2. BACTERIOPHAGE STRAINS employed in this study are listed below. Recombinants derived during the course of this work are described in the text and are not included here.

Designation and genotype	Source	Reference
λ^+	WJB	Hershey (1971)
λ *c*Its857	WJB	Sussman & Jacob (1962)
λ *c*I26	WJB	Meselson (1964)
λ *c*II28	WJB	Kaiser (1957)
λ *c*III67	WJB	Kaiser (1957)
λ *vir* (v_2, v_1v_3)	WJB	Jacob & Wollman (1954)
λ *imm*434	WJB	Kaiser & Jacob (1957)
λ *imm*434 *c*II28	WJB	
λ *imm*434 *c*III67	WJB	
λ *imm*21	WJB	Liedke-Kulke & Kaiser (1967)
λ *Nam*7 *Nam*53	WJB	Campbell (1961)
λ *Nam*7 *Nam*53 *nin*R5	WJB	Court & Sato (1969)
λ *pbio*1	NEM	Manly *et al.* (1969)
$\phi 80^+$	WJB	Matsushiro (1963)
$\phi 80$ *vir*	WJB	
λ *h*$^{+\phi 80}$ *att*$^{\phi 80}$ *c*Its857 *nin*R5	NEM	NEM unpublished
λ *h*$^{-\phi 80}$ *att*$^{\phi 80}$ *nin*R5	NEM	Brammar *et al.* (1974)
λ *trp*51 (λ *h*$^{-\phi 80}$ *att*$^{\phi 80}$ *trp*DE *nin*R5)	WJB	Brammar *et al.* (1974)
λ *trp*51 *Nam*7 *Nam*53 *nin*$^+$	WJB	WJB unpublished
λ *trp*$^+$51 (λ *h*$^{-\phi 80}$ *att*$^{\phi 80}$ *trp*ABCDE *nin*R5)	WJB	WJB unpublished
λ *pgal*8 *nin*R5	WJB	Feiss *et al.* (1972)

BACTERIOPHAGE STRAINS (Cont'd)

Designation and genotype	Source	Reference
λ plac5 cIts857 ninR5	NEM	Ippen et al. (1971)
λ plac5 (sRI λ 2, att λ) ▽ sR1 λ 3° cIam4° 5°	NEM	NEM unpublished
λ amp4 cIts857 Sam7	WJB	Hayes (1976)
λ b538 sRI λ 3° imm434 sRI λ 4° 5° (λ vector 607)	NEM	Murray et al. (1977)
λ int4 red3 gam am210 chiD123	NEM	Stahl et al. (1975)
λ (sRI λ 1-2) ▽ imm21 ninR5 shn λ 6° (λ vector 540)	NEM	Borck et al. (1976)

In vitro recombinant phages:

(λ vector and restriction enzyme used)

λ trpA^{L} 1	(λ vector 540:HindIII)	NEM	Hopkins et al. (1976)
λ trpC^{L} 2	(λ vector 540:HindIII)	NEM	Hopkins et al. (1976)
λ trpE^{L} 17	(λ vector 540:HindIII)	NEM	Hopkins et al. (1976)
λ trpABC^{L} 19	(λ vector 540:HindIII)	NEM	Hopkins et al. (1976)
λ trpCDE^{r} 1710	(λ vector 540:HindIII)	NEM	Hopkins et al. (1976)
λ trpA^{+r} (BCDE) ▽ BE9	(λ vector 574:HindIII)	NEM	Hopkins et al. (1976)
λ (sR1 λ 1-2) ▽ gam am210 cIts857 sRI λ 4° nin R5 sRI λ 5°	(vector 569: ——)	NEM	Borck et al. (1976)

In vitro recombinant phages (Cont'd)

		Source	Reference
λ*supD*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ*supE*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ*supF*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ*supF*	(λ vector 540:*HindIII*)	NEM	Borck *et al.* (1976)
λ*cysB*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ*hsdS*,*hsdM*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ*thyA*	(λ vector 540:*HindIII*)	NEM	Borck *et al.* (1976)
λ*tna*	(λ vector 540:*HindIII*)	NEM	Borck *et al.* (1976)
λ*hisB*	(λ vector 540:*HindIII*)	NEM	Borck *et al.* (1976)
λ*hisG*	(λ vector 540:*HindIII*)	NEM	Borck *et al.* (1976)
λ*lig*	(λ vector 569:*EcoRI*)	NEM	Borck *et al.* (1976)
λ D13	(λ vector 569:*EcoRI*)	Dr K. Borck cited in Borck *et al.* (1976)	
λ*gln*	(λ vector 722:*HindIII*)	NEM	NEM unpublished
λ*polA*	(λ vector 722:*HindIII*)	NEM	NEM unpublished
	λ vector 722 is described in		Murray *et al.* (1977)

Other bacteriophages used in this work:

MS2	RT	
T6	WJB	
T4	GGW	Benzer (1955)
T4 NG19 (gene34*am*)	GGW)	From the Collection of
T4 A1 (gene32*am*)	GGW)	Professor N. Symonds
P1*kc*	WJB	Lennox (1955)

3. GROWTH MEDIA & DILUTION BUFFERS

All media and solutions were sterilised by autoclaving at 15lb/sq.in. for 15 min. before use.

L broth (Lennox, 1955) was the rich medium used for liquid cultures.

Difco Bacto Tryptone	10g
Difco Bacto Yeast extract	5g
NaCl	5g
Glucose	1g

Distilled water added to final volume of 1 litre

Adjusted to pH 7.2 before autoclaving.

L-agar

L-broth (as above)

Solidified with Difco agar 10g/ℓ

BBL agar (Parkinson, 1968)

Trypticase (Baltimore Biological Laboratories)	10g
NaCl	5g
Difco Bacto agar	10g

Distilled water added to final volume of 1 litre

BBL soft-agar

BBL agar containing only 6.5g/ℓ Difco Bacto agar.

GROWTH MEDIA & DILUTION BUFFERS (Cont'd)

Spizizen minimal agar (Spizizen, 1958)

New Zealand agar 15g

Distilled water added to final volume of 800ml

After autoclaving the following additions were made:

5x Spizizen salts 200ml

20% (w/v) solution of required carbon source 10ml

ACH agar

Spizizen minimal agar (with salts & carbon source)

Difco Bacto Acid-hydrolysed Casein (vitamin-free) added to 0.05% (w/v) after autoclaving.

Minimal soft-agar (for use with minimal & ACH agar plates)

New Zealand agar 7g

Distilled water added to final volume of 1 litre.

MacConkey agar

Oxoid Lactose MacConkey agar (No 3) 51.5g

Distilled water added to final volume of 1 litre.

or

Difco Bacto MacConkey agar base 40g

20% (w/v) solution of required carbon source 50ml

Distilled water added to final volume of 1 litre.

GROWTH MEDIA & DILUTION BUFFERS (Cont'd)

BBL-XG (Horowitz et al., 1964)

5-bromo-4-chloro-3-indolyl-β -D-galactoside (XG)

2.5mg/ml in dimethylformamide (DMF) 16ml

Added to molten BBL agar at about 50°C 1 litre

(The stock XG solution in DMF was stored at -20°C).

BBL-PVA agar

Polyvinyl alcohol 7.5g

Added to BBL agar (before autoclaving) 1 litre

BBL-PVA soft agar

Polyvinyl alcohol 7.5g

Added to BBL soft-agar (before autoclaving) 1 litre

5x Spizizen salts

$(NH_4)_2SO_4$	10g
K_2HPO_4	70g
KH_2PO_4	30g
Tri-sodium citrate.$2H_2O$	5g
$MgSO_4.7H_2O$	1g

Distilled water added to final volume of 1 litre

20x Vogel Bonner salts

$MgSO_4.7H_2O$	4g
Citric Acid	40g
K_2HPO_4	200g
$Na(NH_4)HPO_4.4H_2O$	70g

Distilled water added to final volume of 1 litre

2ml chloroform was added as a preservative.

GROWTH MEDIA & DILUTION BUFFERS (Cont'd)

Phage dilution buffer

KH_2PO_4	3g
Na_2HPO_4	7g
NaCl	5g
$MgSO_4$, 0.1M aq. solution	10ml
$CaCl_2$, 0.1M aq. solution	10ml
Gelatin, 1% (w/v) aq. solution	1ml

Distilled water added to final volume of 1 litre.

Bacterial buffer

As phage dilution buffer omitting the gelatin.

4. ENZYMES AND CHEMICALS

Pancreatic DNAase and RNAase were obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

Lysozyme (GradeI egg-white enzyme) was purchased from Sigma Chemical Co., St Louis, Mo.63178, U.S.A.

T4 polynudeotide ligase and electrophoresis grade agarose was purchased from Miles Laboratories Ltd, Stoke Poges, Slough, Bucks, U.K.

Caesium chloride was obtained from B.D.H. Ltd, Poole, Dorset. U.K.

Whenever possible reagents used were of A.R. grade.

EcoRI restriction endonuclease was prepared as described in section 5(s).

REndo AvaI was a gift of Mr S.G. Hughes.

REndo BamHI was generously provided by Mr D. F. Ward.

REndo HindIII was provided by Mr R. Thompson and also by Mr K. Kaiser.

REndo HindII + III unfractionated mix was a gift of Mr R. Thompson.

5. METHODS

(a) Plating cell suspensions

Cells were diluted 5 fold from a fresh overnight culture into L-broth and harvested in mid-logarithmic phase (after 2 hours shaking at 37°; corresponding to a cell density of approximately $5 \times 10^8 ml^{-1}$). The cells were resuspended in the same volume of 1mM $MgSO_4$ and stored at 4°C.

(b) Phage titration

Phage stocks were serially diluted into phage buffer. A 0.1ml aliquot of an appropriate dilution was added to 0.1ml of plating cells and allowed to adsorb for 15 min. at room temperature. 2.5ml molten BBL soft-agar were then added, and the mixture immediately poured onto a BBL agar plate. After 12 hours or overnight incubation at the required temperature (usually 37°C), the plaques were scored and counted.

(c) Preparation of high titre phage stocks

A well isolated single plaque was picked and transferred to 1ml of phage buffer containing a drop of chloroform, and vortexed to release the phages into the buffer. 0.1ml of this suspension was adsorbed to 0.2ml fresh plating cells, mixed with 2.5ml BBL soft-agar and overlaid onto a fresh L agar plate. Once confluent lysis was observed (usually after 6-8 hours incubation at 37°C), the soft agar was harvested, combined with 3ml L broth and several drops of chloroform, and the mixture vigorously vortexed for 30 seconds. Cell debris, agar and chloroform were removed by centrifugation, the supernatant decanted and stored at 4°C over a drop of chloroform in a bijoux bottle.

(c) Preparation of high titre phage stocks (Cont'd)

To avoid recombination with the bacterial trp genes, stocks of trp phages carrying trp mutations were prepared (wherever possible) on hosts of the same trp genotype or carrying a (tonB-trpAE) deletion.

(d) Phage crosses (after Murray et al. 1973)

Freshly prepared plating cells, usually of strain W1485 or QR47, were infected with a mixture of the two phages each at a multiplicity of 5 p.f.u. (plaque-forming units) per cell. After 15 min. adsorption at room temperature, unadsorbed phages were removed by centrifugation and the infected cells resuspended at a 100-fold dilution in L broth. Infected cultures were grown at 37°C with vigorous aeration for 2 hours, and the cultures treated with chloroform. Recombinant phages were selected on appropriate strains and purified as described for individual crosses.

(e) Selection of phage resistant mutants

A high titre lysate (10^{10}-10^{11} p.f.u./ml) of a virulent phage of the host-range to which resistance was required was prepared. 0.1ml of this lysate and 0.1ml freshly prepared plating cells were mixed and allowed to stand for 15 min. to adsorb the phage. The mixture was then spread onto a dry L agar plate and incubated overnight at 37°C. Several single colonies from this plate were then purified by streaking out onto a fresh L agar plate and purified single colonies tested as follows: Each colony was picked into a drop of bacterial buffer. A loopful of the suspension was streaked once across the surface of a dry BBL agar

(e) Selection of phage resistant mutants (Cont'd)

plate. A known sensitive and a known resistant strain were included as controls. When the streaks were dry, a loopful of the virulent phage was cross streaked over the bacterial streaks and allowed to dry. After overnight incubation at 37°C the clones were scored. Phage resistant strains were unaffected by the presence of challenging phage, whereas sensitive or immune cells show a clear area of lysis.

(f) Lysogen construction

Cells were picked from the turbid area of lysis produced after overnight incubation (at the appropriate temperature) by a spot of phage suspension on soft agar seeded with the required host, and streaked for single colonies on L agar.

Individual colonies were tested for lysogeny by streaking a suspension of the colony against streaks of indicator phages (from suspensions of 10^6-10^7 p.f.u./ml) onto a dry BBL agar plate, and scoring after overnight incubation.

Lysogens of a phage carrying imm$^{\lambda}$ were immune to λ cI26 but sensitive to λ vir. imm$^{\phi 80}$ lysogens were $\phi 80^+$ immune but $\phi 80$ vir sensitive. Lysogens carrying imm21 or imm434 were sensitive to λ vir but immune to λ imm21c or λ imm434c respectively.

Heteroimmune helper λ^+ phage was included in the original phage spot where a lysogen of an integration deficient phage was required; the double lysogens selected by the above procedure, and checked for both immunities.

(g) Induction of a lysogen by ultraviolet light

Fresh plating cells were prepared as described in section 5(a). Aliquots of 1ml were pipetted into an empty petri-dish and exposed to 400 ergs/mm^2 ultraviolet radiation (60 secs. under a lamp delivering 7 ergs/mm^2/sec in this case). The suspension was then diluted 5 fold into fresh L broth in a light-proof culture bottle (to avoid photo-reactivation repair), and grown for 2 hours at 37°C. Surviving cells were lysed by the addition of several drops of chloroform, vortexed, and the cell debris removed by centrifugation. The supernatant was titred for free phage.

(h) Complementation of λ clear plaque mutants (After Kaiser, 1957)

Mutants in each of the three λ clear plaque complementation groups, cI, cII and cIII were used to test the presence of these genes in uncharacterised phages. (The rare class of clear plaque mutants, c^Y, were not considered.)

A well dried BBL agar plate was overlaid with 0.1ml plating cells in 2.5ml molten BBL soft agar. Dilutions of the test phage containing 10^7, 10^8 and 10^9 p.f.u./ml were applied as three drops near the edge of the tilted plate and allowed to run down the surface in three broad streaks, and to dry. Similar dilutions of a standard λ clear mutant were cross-streaked at right-angles to the original streaks. After overnight incubation at 37°C the plates were scored. The streaks caused clearing of the bacterial lawn except where two complementing clears crossed; the turbid area produced indicated the growth of lysogens as a result of complementation.

(i) Measurement of restriction ratios

The titre of phages propagated on the non-restricting host, divided by the titre on the restricting strain, is defined as the restriction ratio.

The K-restriction ratio (R_K) was estimated as the titre of unmodified .0 phages (prepared by passage through the non-restricting host : 803) on the K-restricting host (C600 or W1485), divided into the titre on the non-restricting host (803).

Similarly, the RI-restriction ratio (R_{RI}) was obtained by dividing the titre of the .K phages (prepared by passage through C600) propagated on 5K, by that on 5K/RI.

(j) Measurement of phage burst size

The average burst size of a phage is operationally defined as the number of progeny phage after one cycle of growth, divided by the number of cells originally infected, and was determined as follows:

0.1ml fresh plating cells of the required host were mixed with an aliquot of a phage dilution calculated to give a multiplicity of infection (m.o.i.) of 0.1-0.3, and allowed to stand for 15 min. to adsorb the phage. 1ml Bacterial buffer was then added and the suspension agitated to ensure mixing. The cells were harvested by centrifugation and the supernatant (containing any unadsorbed phage) was decanted and discarded. The infected cells were then resuspended in 1ml Bacterial buffer, mixed, and diluted 10^5 fold into prewarmed L broth at 37°C. The suspension was thoroughly mixed, an aliquot titred for infectious centres, and returned to a shaking water bath at 37°C for 90 min. At the end of this time

(j) Measurement of phage burst size (Cont'd)

several drops of chloroform were added, the mixture vortexed to ensure cell lysis, and titred for phage. The burst size is given by the total phage burst divided by the number of infectious centres.

(k) Complementation of E.coli auxotrophies by λ-transducing phages

These tests are based on the observation of Franklin (1971), that λ trp transducing phages form "Trp^+-plaques" when grown on a trp^- host on a medium lacking tryptophan. The "Trp^+-plaques" are characterised by a circle of lysis surrounded by a ring of growth stimulated by tryptophan feeding. In the case of turbid phages the centre of the plaque is studded with colonies of Trp^+-lysogens. Clear plaque mutants often elicit a poor response in this test, and are more difficult to score, but addition of one drop of L broth to the top agar layer improves the response by enhancing growth of the bacterial lawn.

Specific tests were performed as follows:

0.1ml plating cells or of a fresh overnight culture in 2.5ml minimal soft agar was overlaid onto an appropriately supplemented minimal agar plate (see below). Tenfold serial dilutions of λ-transducing phages were then spotted onto the top layer (0.01ml each), and the spots allowed to dry. Plates were incubated at 37°C (or 32°C for λ cIts857 derivatives). Complementation was scored after 36-72 hours. Media used in these tests for the listed auxotrophies are given below:

trp : ACH agar with glucose as carbon source.

(k) Complementation of E.coli auxotrophies by λ -transducing phages (Cont'd)

tna : Minimal agar with glycerol as carbon source, containing 100 μg/ml 5-methyltryptophan and 2 μg/ml Indole.

his, cys, thy and gln : Minimal agar with glucose as carbon source, plus 40 μg/ml any other amino acids required by the host.

(l) Staining test for penicillinase

Phage were plated for single plaques on BBL-PVA agar plates using BBL-PVA soft agar as top layer and C600 as the bacterial host, as described in 5(b), and incubated overnight at 37°C.

Plates were stained with 5ml of a 4 fold dilution of stock iodine solution (0.32N I_2 in 1.2M KI) in distilled water, for about 30 secs. when uniform deep brown staining was achieved. The stain was poured away and the agar surface washed with 5ml 2% (w/v) aqueous solution of benzyl-penicillin. A further 5ml benzyl-penicillin solution was poured onto the plate and allowed to diffuse into the agar for 2 mins. before being drained away. Plaques with associated penicillinase activity were recognised by clearing of the intense blue stain of the surrounding agar within 15-20 mins.

(m) Conjugation: plate matings

Freshly prepared plating cells of the donor (Hfr) and the recipient (F^-) strains were cross streaked onto a dry minimal agar plate appropriately supplemented to select the desired ex-conjugant and containing 40 μg/ml streptomycin, when this was the counter-selective agent. Plates were allowed to dry and incubated overnight at 37°C. Desired recombinant clones appeared as colonies in the second cross-streak downstream of the intersection, and these were purified by streaking out onto similar selective plates and incubating overnight at 37°C. Single purified colonies were then grown up in L broth and tested for the required genetic markers.

(n) Generalised transduction by phage P1kc

P1kc lysates were prepared on the desired host strain as in 5(c) using L agar plates made up to 2.5mM in $CaCl_2$.

0.05ml of sterile 50mM $CaCl_2$ solution, 0.2ml fresh overnight culture of recipient cells and 0.05ml of a lysate of P1kc (diluted to 10^{10} p.f.u./ml) were added to 1ml L broth, and incubated at 37°C for 20 mins. to allow phage adsorption. The cells were then collected by centrifugation and resuspended in 1ml sterile 0.1M trisodium citrate, pH7. The suspension was diluted 2 fold in L broth and grown for 4-6 hours at 37°C to allow segregation of phenotypes. When selection was for loss of an auxotrophy the last step was omitted and 0.1ml aliquots of cells spread directly onto selective agar plates. In the case of selection for phage resistance, the segregation lag is critical; 0.01ml and 0.1ml aliquots of the broth grown cells were

(n) Generalised transduction by phage P1kc (Cont'd)

spread onto L agar plates seeded with 10^9 p.f.u. of the selective phage and incubated overnight at 37°C. The transductant clones were purified by streaking out onto L agar plates, and single isolated colonies grown up in L broth and tested for the genetic markers of interest.

P1kc occasionally forms stable lysogens, but these can be detected by the restriction ability confered by the prophage. Transductants were tested by parallel titrations of λ.K on C600 and on the transductant strains; the efficiency of plating was the same on both strains (e.o.p. =1) for non-lysogens. (P1 lysogens would reduce the e.o.p. by several orders of magnitude).

(o) Construction of λ -transducing phages carrying known trp alleles

λtrp lysogens of attλ-deleted host strains carrying the trp allele desired on the phage were constructed by rec mediated recombination via trp homology as follows:

Where the phage complemented the host trp lesion, trp^+ lysogens were selected by spotting 0.01ml of serial dilutions of the λtrp phage onto minimal top layer containing 0.1ml fresh overnight culture of the attλ ,trp strain, overlaid onto an ACH agar plate, and the single colonies produced were purified by streaking onto fresh ACH plates.

When complementation was not possible, lysogens were prepared as described in 5(f) except that clones were selected and purified on L agar plates seeded with 10^9 p.f.u. each of two

(o) Construction of λ -transducing phages carrying known trp alleles (Cont'd)

heteroimmune clear phages of different host ranges (usually λ c and λ h$^{\phi 80}$ c).

Lysogens were induced by ultraviolet radiation as already described, or in the case of those carrying the cIts857 marker, heat induced as follows: An exponential culture grown in L broth at 30°C with good aeration, was transferred to 42°C for 15 min. and then grown for a further 2 hours at 37°C with shaking; at the end of this time the culture was lysed by the addition of several drops of chloroform and plated for single plaques of induced phage with a str^{S} host on BBL agar.

Isolated single plaques were picked with a toothpick and stabbed into top layer seeded with a trp^{-},str^{R} indicator strain on ACH agar containing 40μg/ml streptomycin. Phages which had acquired the mutation fail to complement trp^{-} strains mutant in the same gene.

(p) Pyrophosphate selection of deletion derivatives of λ trp phage (Parkinson & Huskey, 1971)

Phage lysates were diluted 50 or 100 fold into 20mM sodium pyrophosphate (adjusted to pH7.0 with phosphoric acid), and incubated at 42°C for 10 mins; the reaction was stopped by diluting samples into phage buffer at room temperature. Aliquots were plated for single plaques on BBL agar containing sodium pyrophosphate added to the bottom agar to 2.5mM final concentration.

(p) Pyrophosphate selection of deletion derivatives of λ trp phage (Parkinson & Huskey, 1971) (Cont'd)

Survivors were screened for deletions entering the trp operon by stabbing plaques from a str^S lawn onto an ACH plate containing 40μg/ml streptomycin, overlaid with a str^R trp^- host in 2.5ml minimal soft agar. Phages which have lost all or part of trp fail to give "trp^+-plaques" on trp AE8,str^R, and were tested further for the presence of individual trp genes.

(q) Large scale liquid lysates of phage

1 litre L broth prewarmed to 37°C was inoculated with 1/20 - 1/50 volume of a fresh overnight culture of C600 (W1485 for $h^{\phi 80}$ phages) and vigorously shaken, to provide good aeration, at 37°C until the O.D. at 650nm reached 0.6 (estimated 3 x 10^8 cells/ml). Phage from a high titre stock were added to a multiplicity of infection of 1.0, taking care not to carry over any chloroform from the stock bottle. The $O.D._{650}$ was then followed, taking readings at 30 min. intervals until the O.D. began to fall (2-4 hours), then every 15 min. until a minimum value was reached. 2ml chloroform were then added and shaken vigorously. The lysate was allowed to stand on ice for 10 mins, and clarified by centrifugation at 10Krpm for 10 mins in an MSE HS18 in a 6 x 250ml capacity rotor. Clarified suspensions show a strong Tyndall effect (usually 2 x 10^{10} - 10^{11} p.f.u./ml) and were titred for free phage.

Alternatively, for convenience, the PDS (preadsorb, dilute, shake) method of Blattner (1976) was used: 0.3ml of a fresh

(q) Large scale liquid lysates of phage (Cont'd)

overnight culture of C600 or the appropriate host was inoculated with a dilution from a single plaque suspension calculated to provide approximately 6.10^5 - 10^6 p.f.u. of phage. 0.3ml of a solution containing 10mM $CaCl_2$, 10mM $MgSO_4$ was then added and the mixture shaken and allowed to stand 20 mins at room temperature to preadsorb. This was used as the inoculum for the desired volume of L broth (100ml - 1 litre) made up to 1mM in $MgSO_4$. The culture was then placed on a shaker at 37°C for overnight incubation with good aeration and subsequently lysed, clarified and titred as in the previous method, without regard for cell density. This method was found to be less reliable than that previously described when used with turbid phages.

(r) Preparation of phage DNA

Phages were recovered, concentrated and purified from large scale lysates as follows:

Bulk liquid lysates were centrifuged at 21Krpm for 3 hours in a 10 x 100ml rotor in an MSE superspeed 65/75, resuspended by gently shaking the pellet with 1/100 original volume of phage buffer overnight and the milky suspension obtained reclarified by spinning at 10Krpm for 10 mins in an MSE HS18 as before. This concentrated phage stock was treated with RNAase and DNAase (10ug/ml each from 1000X stock solutions stored at -20°C) and incubated at room temperature for 1 hour to digest all the remaining free host nucleic acid.

(r) Preparation of phage DNA (Cont'd)

The digest was made up to 41.5% (w/v) in CsCl by adding 0.709g solid CsCl per g phage suspension, was thoroughly mixed and then centrifuged to equilibrium at 27Krpm for 24 - 36 hours in a swing-out titanium rotor in an MSE superspeed 65/75 (Kaiser & Hogness, 1960). The phage band was collected in drops from a hole pierced in the bottom of the tube, diluted 2 - 5 fold with fresh clarified 41.5% (w/v) CsCl solution and rebanded, this time at 33Krpm for 24 hours and collected as before.

The purified phage were diluted with phage buffer to give an estimated 5×10^{12} p.f.u./ml and dialysed for 1 - 2 hours against 10mM Tris-HCl, pH8.0, 1mM EDTA. DNA was extracted by gently rolling for 2 - 3 mins with an equal volume of freshly distilled phenol, and collecting the aqueous phase (Kaiser & Hogness, 1960). This step was repeated twice more, or until there was no longer a white gelatinous precipitate at the aqueous : phenol interface. The viscous DNA solution obtained was dialysed exhaustively against 10mM Tris-HCl, pH8.0, 1mM EDTA (minimum of 4 changes in 24 hours). DNA preparations were assayed by determination of the O.D. at 260nm (an OD_{260} of 1.0 corresponding to a concentration of 50ug/ml). The ratio OD_{260}/OD_{280} was also routinely determined and gave values approaching 2.0 for protein-free solutions. Values of 1.7 or less indicate an unacceptably high protein content and require re-extraction with phenol. Preparations were stored in sterile screw-capped plastic tubes in a refrigerator (4 - 6°C) for several years without deterioration.

(s) Preparation of EcoRI (After Yoshimori, 1971)

Extraction buffer contains:-

10mM Potassium phosphate pH7.0,

1mM EDTA,

7mM 2-Mercaptoethanol,

in distilled water.

Frozen cells (100g) were thawed and suspended in 250ml extraction buffer in a glass beaker and sonicated in 5 x 30 sec. bursts with intermittent periods of 1 - 2 mins to allow cooling (the suspension was stood in an ice-bath throughout the procedure). The total extract was clarified by low speed centrifugation (10Krpm in an MSE HS18 for 15 mins) and then at high speed (35Krpm in an 8 x 50ml rotor in a SS65/75) for 1 hour. The (230ml) supernatant was decanted into a 2l beaker and 100ml 5% (w/v) Streptomycin sulphate solution in extraction buffer added slowly with constant stirring. After 15 mins stirring the precipitate was removed by centrifugation (10Krpm for 10 mins in a 6 x 250ml rotor in an MSE HS18) and solid ammonium sulphate slowly added to the supernatant to 50% saturation (105g to 340ml). After stirring for 30 mins the precipitate was recovered by centrifugation (10Krpm, 10 mins in an MSE HS18), dissolved in 45ml extraction buffer and dialysed exhaustively against extraction buffer. The ammonium sulphate fraction (77ml) was then made up to 0.35M in NaCl by the addition of 7.4ml 4M NaCl stock solution.

A 15cm x 3cm diameter column of Whatman P11 phospho-cellulose was prepared after pre-cycling the resin in 0.1M HCl and 0.1M NaOH

(s) Preparation of EcoRI (After Yoshimori, 1971) (Cont'd)
with extensive washings and final equilibration in 10X extraction buffer, then extraction buffer containing 0.35M NaCl according to the manufacturer's instructions. The ammonium sulphate fraction was loaded onto the column at a flow rate of 2 - 3 column volumes/hour, washed with 50ml 0.35M NaCl in extraction buffer and eluted with a linear gradient from 0.35 - 0.8M NaCl in 600ml total volume extraction buffer at the same flow rate, collecting 10ml fractions.

Fractions were assayed by mixing 20ul aliquots with 0.5ug λ DNA in EcoRI restriction buffer and allowing digestion to proceed for 1 hour at 37°C, followed by analysis of 1% agarose slab gels as described in the next sections. Active fractions show discrete DNA bands in assay digests.

Active fractions (from the 0.5M NaCl region of the elution gradient) were pooled, dialysed against extraction buffer and loaded at a flow rate of 20ml/hour onto a 5 x 1cm diameter column of DEAE-cellulose (Whatman DE-52) equilibrated with extraction buffer. The column was washed with 20ml 0.15M NaCl in extraction buffer and eluted with a 200ml linear gradient of 0.15M to 0.50M NaCl in extraction buffer at a flow rate of 10ml/hour, collecting 4ml fractions. Fractions were assayed as before and active fractions (around 0.3M on the gradient) again combined, and dialysed against extraction buffer.

The enzyme was concentrated by adsorption to a small column (2cm x 1cm diameter) of DE52, equilibrated as before and eluted

(s) Preparation of EcoRI (After Yoshimori, 1971) (Cont'd)

with a single step of 0.5M NaCl in extraction buffer, collecting 1ml fractions. An equal volume of glycerol was added to each of the active fractions and carefully mixed by stirring. The enzyme preparation can be stored indefinitely in this form at -20°C without loss of activity.

(t) Digestion of DNA by site-specific endonucleases ('Restriction' digests)

0.5 - 1.0ug DNA was used for analytical purposes and 1 - 10ug for digests subsequently to be used in ligation experiments. Reactions were generally carried out at a DNA concentration of 25 - 50ug/ml depending on the initial concentration of DNA and enzyme samples used.

10X restriction buffer contained:
(0.1M Tris-HCl pH7.5 (0.01M final)
(0.1M $MgCl_2$ (0.01M final)
(0.1M 2-mercaptoethanol
((0.01M final)

Predetermined volumes of 10X restriction buffer, distilled water, DNA (in 10mM Tris-HCl, pH8.0, 1mM EDTA) and enzyme* were dispensed onto parafilm, using Corning micropipettes or Drummond 'microcaps', mixed, and the appropriate volume of 1M NaCl added to bring the final concentration to 0.1M for EcoRI, or 0.05M for HindIII or Hind II & III digests. Incubation was at 37°C in sealed capillary tubes when volumes of up to 50ul were used, and in sterile screw-capped plastic tubes for larger scale reactions. Digestion was stopped by heating to 70°C for 10 mins in experiments

(t) Digestion of DNA by site-specific endonucleases ('Restriction' digests) (Cont'd)

where digests were used as substrates for ligation, or by adding 1ul 1M EDTA pH9 per 50ul digestion mix prior to storage. Routine analytical digests were loaded directly onto agarose gels without stopping the reaction.

* The volume of enzyme fraction required for each digest was deduced from the 'titre' of that particular preparation determined in a trial experiment. (The titre is defined as the smallest volume of enzyme fraction required to yield a total digest of 1ug λ DNA in 1 hour at 37°C.)

(u) Electrophoretic analysis of DNA through agarose gels (Sharp et al. 1973)

A continuous buffer system, using E buffer freshly diluted from a 10X E buffer stock solution was used.

10XE buffer contained:

Tris	48.4g/l	(1 X E: 0.04M final)
Sodium acetate.$3H_2O$	27.2g/l	(1 X E: 0.02M final)
EDTA	3.7g/l	(1 X E: 0.001M final)

1% agarose was prepared by refluxing 2.5g agarose with 250ml 1XE buffer in a Quickfit 500ml glass balloon for 1 - 2 mins. after reaching the b.p. and subsequently cooling until the flask could comfortably be held by hand before pouring. Gels were cast in 40 X 16 X 0.3cm moulds consisting of two glass plates

(u) Electrophoretic analysis of DNA through agarose gels (Sharp et al. 1973) (Cont'd)

separated by lucite spacers and sealed with paraffin grease. One surface in contact with the gel was precoated with 2.5ml 0.2% agarose in distilled water baked onto the surface, to anchor the gel and prevent slippages during electrophoresis. 10 or 16 sample wells were formed round perspex combs, the wells extending the full width of the gel.

Samples of 20 - 30ul were mixed with 5 - 10ul loading buffer containing 10% Ficoll in 0.1XE buffer, 0.025% bromophenol blue (BPB), or in early experiments: 50% glycerol in 0.5XE Buffer, 0.025% BPB, and loaded into individual sample wells. The slab was stood in the lower reservoir of 1XE buffer and a wick of Whatmans 3MM paper saturated in 1XE buffer provided electrical contact from the top of the gel to the upper reservoir. Electrophoresis was at a current of 40mA, at 220 - 240 Volts for 18 hours, when the blue dye had migrated over $\frac{3}{4}$ distance down the gel. The gel was stained by soaking for 15 - 20 mins. in excess 2.5ug/ml ethidium bromide solution and washed for 30 - 45 mins. in distilled water before photographing under ultraviolet light on Ilford FP4 film (4X red filter) which was developed for 9 mins. at 18°C in Microphen (Ilford Ltd., Ilford, Essex, U.K.).

A quick method for electrophoretic analysis of restriction digests was used when accurate comparative data were not required (e.g. when assessing extent of reaction in enzyme titrations, or assaying column fractions during enzyme preparations). This

(u) Electrophoretic analysis of DNA through agarose gels

(Sharp et al. 1973) (Cont'd)

employed a Shandon electrophoresis apparatus with eight 15 X 0.6cm diameter cylinder gels (held in place by nylon mesh and rubber bands) or, a twenty cylinder improvised apparatus for multiple samples. Samples of up to 50ul were applied and electrophoresis was carried out for 3 hours with an applied current of 5mA per tube. Staining and visualisation of DNA by ultraviolet light was performed as for slab gels.

(v) Preparation of gradient acrylamide gels (Jeppensen, 1974)

The gel former was assembled from two glass plates (20 X 16cm) separated on three sides by lucite strips 0.15cm thick and the seal effected by a thin layer of high vacuum silicone grease on contact surfaces, and held in place by bulldog clips. A small (about 1mm) bore teflon tube sealed into the bottom of the mould was used to pump in the gradient.

The following solutions were prepared and degassed in a vacuum desiccator:

7.5% acrylamide

5.0ml	30% acrylamide, 0.8% NN'-bis-methylene-acrylamide (stock solution).
2.0ml	10XE (as above).
10.0ml	50% sucrose (to stabilise gradient)
3.0ml	distilled water
20.0ml	7.5% acrylamide, 0.2% bis (final).

(v) Preparation of gradient acrylamide gels (Jeppensen, 1974) (Cont'd)

3.5% acrylamide

2.4ml	30% acrylamide, 0.8% NN'-bis-methylene-acrylamide (stock solution)
1.6ml	1% NN'-bis-methylene-acrylamide
2.0ml	10XE buffer
4.0ml	50% sucrose
10.0ml	distilled water
20.0ml	3.5% acrylamide, 0.175% bis (final)

stacking gel (acrylamide component)

1.7ml	30% acrylamide, 0.8% NN'-bis-methylene-acrylamide (stock solution)
1.2ml	1% NN'-bis-methylene-acrylamide
0.2ml	10XE buffer
6.9ml	distilled water
10.0ml	5% acrylamide, 0.25% bis

1ml 10% N,N,N',N'-tetramethylethylenediamine (TEMED, Eastman Kodak Ltd, London) and 1ml 10% ammonium persulphate were freshly prepared as polymerising agents.

100ul TEMED and 50ul persulphate were added to the 7.5% acrylamide solution and 25ul TEMED and 50ul persulphate added to the 3.5% solution, and stirred taking care not to cause bubbles

(v) Preparation of gradient acrylamide gels (Jeppensen, 1974) (Cont'd)

and reaerate the solutions. These were then poured into the two chambers of a gradient maker arranged to give a linear gradient from 3.5% to 7.5% acrylamide, pumping the less dense end of the gradient into the mould first, under 2 - 3ml distilled water (this allows the top of the gel to level out more evenly). A peristaltic pump set to deliver about 5ml/min. was used and 2ml 50% sucrose solution pumped in after the gradient to prevent polymerisation in the tubes and hence blockage of the pump and supply tube. The gel was completely set after 30 mins, when the upper layer of water was poured away and the top of the gradient washed twice with 2ml unpolymerised stacking gel mix.

The sample well former (13 or 16 slots) was placed in position above the gradient. The remaining stacking gel mix was stirred with an equal volume of 1% agarose in 0.2XE buffer (refluxed and cooled to 50°C), 200ul 10% TEMED and 200ul 10% persulphate and quickly and carefully poured onto the top of the gradient level with the slot former using a 10ml pipette and bulb dispenser (unpolymerised acrylamide and bis. are both potent neurotoxins which are readily absorbed through the skin). After a further 20 - 30 mins. when the stacking gel was set, the slot former was removed and the wells washed and filled with 0.1XE buffer. Samples (up to 20ul) were prepared and loaded as before. Electrophoresis, staining, washing, visualisation and photography were performed exactly as for agarose gels.

(w) Preparation of Escherichia coli DNA (Adapted from Marmur, 1961)

A 500ml L broth liquid culture of the desired strain in late logarithmic growth phase (O.D. at 650nm of about 1.0) was harvested by centrifugation at 4°C and resuspended in 12ml 25% sucrose in 50mM Tris-HCl, pH8.0 . Lysozyme (1.7ml freshly prepared solution containing 10mg/ml in 0.25M Tris-HCl pH8.0, 0.25M EDTA pH8.0) was added, shaken gently for 80 secs. at 37°C and then placed on ice for 5 mins. After this time, 6.5ml 0.25M EDTA pH8.0, was added and the cell suspension left for a further 5 mins. on ice to complete lysis.

13.5ml Triton solution (2% v/v Triton X-100 in 62.5mM EDTA, 50mM Tris-HCl pH8.0) were then added and left a further 20 mins on ice. The volume (now about 34ml) was made up to 1M in perchlorate by the addition of 8ml 5M $NaClO_4$ to dissociate protein from the nucleic acid. The entire mix was then shaken with an equal volume of chloroform:isoamylalcohol (24:1 v/v) in a stoppered flask for 30 mins.

Phases were separated by spinning at 8Krpm for 5 mins and the upper aqueous phase carefully removed with a sterile pasteur pipette. The volume was measured and gently overlaid with two volumes 95% ethanol in a 250ml glass beaker, and gently stirred with a clean glass rod to pull out the DNA threads. Excess fluid was drained away by pressing the glassy spooled nucleic acid against the side of the vessel, and then the threads were stirred and dissolved in 20ml dilute SSC (15mM NaCl, 1.5mM sodium citrate, pH7.0).

(w) Preparation of Escherichia coli DNA (Adapted from Marmur, 1961) (Cont'd)

The homogeneous solution obtained was made up to standard SSC concentration (0.15M NaCl 0.015M sodium citrate, pH7.0) with 10XSSC stock solution, and again shaken with an equal volume of chloroform : isoamylalcohol for 15 mins. Extractions were repeated several times until very little protein appeared at the interface between phases. The supernatant from the final deproteinisation step was precipitated with 2 volumes 95% ethanol, the nucleic acid wound out onto a glass rod and dispersed in dilute SSC (0.5 X supernatant volume) as before.

The solution was incubated with RNAase (from a concentrated solution containing 20mg/ml in 0.15M NaCl, pH5.0, preheated to 80°C for 10 mins, to inactivate any contaminating DNAase) at a final concentration of 50ug/ml, for 30 mins at 37°C.

The preparation was then extracted twice by rolling gently with an equal volume of freshly distilled phenol (equilibrated with an equal volume of 0.5M Tris-HCl, pH8.0) followed by extensive dialysis against 10mM Tris-HCl pH8.0.

The concentration of DNA was assayed spectrophotometrically exactly as for phage DNA (previously described).

(x) Ligation of 'restriction' fragments of DNA

Restricted DNA and receptor DNA were mixed in equal proportions by weight and diluted to a final concentration of 20ug/ml with 10mM Tris-HCl pH7.5 in a plastic screw-capped tube of 2ml capacity. Then 0.1 volume 1M NaCl was added and 0.1 volume 10X T4 ligase cocktail.

(x) Ligation of 'restriction' fragments of DNA (Cont'd)

T4 ligase cocktail		final concentration
1M Tris-HCl pH7.5	66ul	66mM
0.4M EDTA pH9.0	2.5ul	1mM
1M $MgCl_2$	10ul	10mM
1M Dithiothreitol	10ul	10mM
0.1M ATP (freshly prepared)	1.0ul	0.1mM

The reaction mixture (usually 0.1 - 0.3ml) was reincubated at 70°C for 10 mins (to reopen any annealed λ cos ends and ensure total dissociation of restriction fragment ends) and transferred to ice. T4 polynucleotide ligase (Miles Laboratories Ltd) was added to a final concentration of 1 unit/ml (2ul/ml), and the mixture incubated at 10°C for 3 - 6 hours, then transferred to an ice bath where it was kept throughout the sampling period. When transfection or transformation assays showed a decline in recoverable plaques or clones per ul reaction mixture (usually after 6 - 8 days) the total mix was subjected to 70°C for 10 mins (to inactivate both ligase and competing nucleases) and stored in the refrigerator at 4 - 6°C until required.

(y) Preparation of E.coli cells competent in DNA uptake, transformation/transfection

(Modified from the procedures of Mandel & Higa, 1970; Lederberg & Cohen, 1974)

It is imperative to use fresh bottle-grown L broth overnight cultures (o/ns) of the desired competent strain to achieve the maximum efficiency (5mls in a half ounce bottle ensures a short lag before growth resumes when subcultured).

(y) Preparation of E.coli cells competant in DNA uptake, transformation/transfection (Cont'd)

An o/n was diluted 50 fold into fresh prewarmed L broth in a flask of at least 5 times that capacity, and grown at 37°C with vigorous aeration on an orbital shaker until the O.D. at 650nm reached 0.65 - 0.70. The cells were chilled on ice for 3 - 5 mins, harvested by centrifugation, resuspended in half the original volume of ice-cold 0.1M $MgCl_2$ solution and harvested again. The cells were then resuspended in 1/20 original volume ice-cold 0.1M $CaCl_2$ solution, kept on ice and used as quickly as possible.

DNA in 0.1ml sterile Ca/SSC (2 volumes 0.1M $CaCl_2$, 1 volume standard SSC : 0.15M NaCl, 0.015M sodium citrate, pH7.0) was added to 0.2ml calcium-treated competent cells, prepared as described above, and incubated at 0°C for 30 mins. The cells were heat-pulsed at 42°C for 2 mins and returned to 0°C for a further 30 mins. After this time aliquots of 0.1ml of the mixture were mixed with 2.5ml BBL soft agar and overlaid onto BBL agar plates for phage plaques or spread directly onto selective plates (L agar containing antibiotics, or supplemented minimal agar with/without antibiotics) for transformant colonies as appropriate.

(z) Heteroduplex techniques and electron microscopy (Simon et al. 1971)

1ml concentrated phage stocks (about 10^{12} p.f.u./ml) were prepared from clarified (10Krpm, 10 mins in an 8X50ml rotor in an MSE HS18 centrifuge) 100ml liquid lysates by harvesting : 3 hour spin at 21Krpm in a 10 X100ml rotor in an MSE SS 65/75:and resuspending the pellet in 1ml phage buffer by gentle pipetting.

(z) Heteroduplex techniques and electron microscopy (Simon et al.1971) (Cont'd)

Stocks were clarified by a 2 min. spin in a bench microcentrifuge at 7Krpm and stored over a drop of chloroform.

Volumes (approximately 40ul) of each of the two phage stocks required for heteroduplex analysis containing $3\text{-}5X10^{10}$ p.f.u. (estimated 1.5 - 2.5ug DNA) were denatured in 0.5ml 0.1M NaOH, 0.02M EDTA by standing at room temperature for 10 mins, then neutralised by the addition of 50ul 1.8M Tris-HCl, 0.2M Tris-base and 0.5ml formamide. Renaturation and heteroduplex annealing was achieved by 2 hours incubation at 27°C, followed by immediate chilling to 0°C to prevent further renaturation and formation of higher aggregates.

50ul of the renatured mixture was diluted into 1ml hyperphase solution (0.1M Tris-HCl, pH8.5, 0.01M EDTA, 50% formamide, prepared within an hour of use) at room temperature and allowed to stand for 2 - 3 mins. 50ul cytochrome c (Sigma Chemical Co. St Louis, Mo. 63178, U.S.A.) solution : 1mg/ml in 20mM Tris-HCl, pH8.5, 2mM EDTA, was then added.

Spreading is very sensitive to draughts or vibrations, so the remaining procedures were carried out under a large draught-excluding hood on a stable surface. 50ul of the DNA and cytochrome c solution was spread onto the surface of 100ml hypophase solution (10mM Tris-HCl, pH8.5, 1mM EDTA, 15% formamide, freshly prepared from concentrated stocks of the constituents within 5 mins of use: dilute aqueous solutions of formamide are very unstable and become

(z) Heteroduplex techniques and electron microscopy (Simon et al.1971) (Cont'd)

acid very quickly), which had been very lightly dusted with talc powder. A fused quartz ramp was used, dipping into the hypophase, for this purpose; the sample was gently introduced from an Eppendorf micropipette to a position on the ramp 0.5 - 1.0cm above the level of the hypophase, and the talc observed to ensure adequate spreading had occurred. After 1 min. a fresh parlodion coated copper grid was gently touched against the surface about 1cm from the hypophase boundary on the ramp and then dipped in stain (5×10^{-5} M uranyl-acetate, 5×10^{-5} M HCl in 90% ethanol, freshly prepared) for 30 secs, washed in 90% ethanol for 10 secs and dried by lightly touching the edge of the grid against a filter paper circle.

Grids were rotary shadowed with platinum vaporised from a Pt wire on a heated filament at an angle of $\tan^{-1} 0.2$ (11.3°), whilst mounted on a motor driven turntable spinning at several hundred rpm.

Stained and shadowed grids were scanned with a Siemens Elmiskop 1A electron microscope at 20,000 times magnification with an 80 KV accelerating voltage. (In cases when the parlodion was found to be unstable on exposure to the electron beam, the grid was removed, coated with carbon from an arc across graphite electrodes and the parlodion dissolved by washing in absolute ethanol for 1 min. Carbon coatings were found to be more robust and withstand the bombardment of the electron beam).

(z) Heteroduplex techniques and electron microscopy (Simon et al.1971) (Cont'd)

Heteroduplex molecules which were extended along one axis were rejected as they could have been stretched. Suitable molecules were photographed directly at 20,000 times magnification and negatives enlarged 5 fold and traced onto large sheets of card. Molecules were measured with a map measurer and the accumulated data statistically analysed.

6. Tests for E.coli genes carried by λ -transducing phages

The complementation tests used for E.coli auxotrophies are described in section (k).

λ hsd_K : single plaques of the putative λ hsd_K phage, and also of the vector λ569 were grown on the non-modifying host 803. Ten-fold serial dilutions of each were then spotted onto a BBL agar plate overlaid with C600 and another with 803, and incubated overnight. The unmodified λ569 is restricted about a thousand-fold, whereas λ hsd_K shows the same titre on both strains.

λ lig and λ lig-lop-11 : double-lysogens of the putative λlig and λ imm21 in N3098 (ligts7) were prepared by spotting 10^6 pfu of each phage onto a lawn of N3098 overlaid on BBL agar, and streaking for single colonies onto L-agar from the turbid area produced. Lysogens were tested for both immunities and for growth on L-agar at 37° and 42°C. Double lysogens containing λlig grew at both temperatures, whereas those containing only the vector λ569 and λimm21 failed at 42°C.

6. Tests for E.coli genes carried by λ-transducing phages (Cont'd)

λD13 and λbio72 : both phages were tested for the Fec$^+$, Feb$^-$ phenotypes conferred by their red$^-$ substitutions, by growth on a recA strain (QR48) and failure to grow on N3098 (ligts7) by comparing spot-titres on BBL agar overlaid with the appropriate host. Vector λ569 is red$^+$ and grows efficiently on both hosts.

λpolA : double lysogens of the putative λpolA and λimm21 helper in KB8 (polA1) were constructed and streaked for single colonies on L-agar containing 0.04% MMS. λpolA allows the strain to grow in the presence of the alkylating agent whereas the vector λ 569 does not.

λpgal8 : this phage produces turbid red plaques on W3350 when grown on galactose-MacConkey agar, whereas control phages give colourless plaques.

λplac5 : produces turbid red plaques on ED8538, or other lacZ$^-$ hosts on lactose-MacConkey agar, whereas lambda wild-type gives colourless plaques.

λsup phages : these were preliminarily checked as allowing the growth of ED8538 (leu am , lacZam , trpA33) on minimal agar supplemented with tryptophan (20 ug/ml), and producing red plaques on this strain when grown on lactose-MacConkey agar; and giving Trp$^+$ plaques on trpC55 (Cam 9870, am10243) on ACH-agar. The λsupE phage gives the weakest response in these tests.

6. Tests for E.coli genes carried by λ-transducing phages (Cont'd)

Confirmatory evidence was obtained by comparing the ability of lysogens of these phages in a trpC55 strain (selected as Trp^+ colonies on ACH agar) to support the growth of various T4am phages:

bacteriophage:-	$T4^+$ wild type	T4 NG19 (gene 34am)	T4A1 (gene 32am)
W3350 (sup°)	+	-	-
CR63 (supD)	+	±	+
C600 (supE)	+	-	+
Ymel (supF)	+	+	-
trpC55 (λsupD)	+	±	+
trpC55 (λsupE)	+	-	+
trpC55 (λsupF)	+	+	-
trpC55 (λ)	+	-	-

RESULTS

CHAPTER 3

CONSTRUCTION AND CHARACTERISATION OF λtrp51 SERIES OF PHAGES

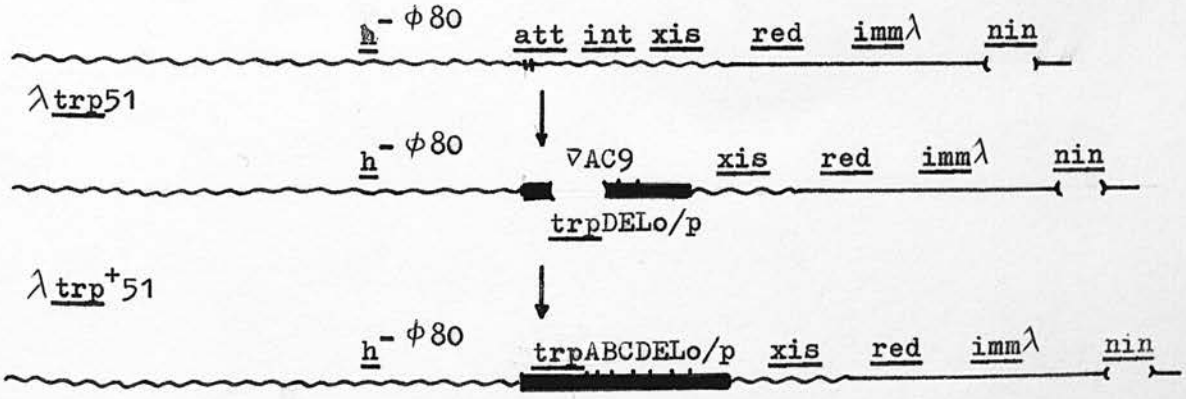
(a) Construction of phages

The non-defective *trp* transducing phage λ*trp*51 was isolated by Brammar *et al.* (1974). It was formed by the classical mechanism of aberrant excision of a $\lambda h^{-\phi 80} att^{\phi 80} ninR5$ lysogen of W3110(*tonB-trp*)▽AC9 and thus carries only the D and E genes of the *trp* operon, and since it exhibits normal repression by tryptophan, the *trp* operator and promoter remain intact; it also retains λ*red* function (Brammar *et al.* 1974) and φ80*xis* (Brammar & Dunbar, personal communication).

$\lambda trp^{+}51$ was induced from a W3110(λ*trp*51) lysogen and selected as a rare plaque-forming phage able to complement *trp*▽AE8, and thus carries the entire *trp* operon (W.J. Brammar, unpublished work).

This information is summarised below:-

λ:φ80 hybrid (NM407.5) parent of λtrp51 series



Wavy lines represent DNA from $\phi 80$, thin straight lines that from λ, and heavy lines bacterial DNA. The extended host range mutation $\underline{h}^{-}\phi 80$ permits the hybrid phage to grow on (tonB-trp)∇ hosts which are resistant to $\phi 80\underline{h}^{+}$, and the phage attachment site $\underline{att}^{\phi 80}$ specifies prophage integration near trp on the host chromosome. The deletion ninR5 reduces the DNA content of the hybrid phage by 5.4% of the lambda genome (Fiandt et al. 1971), thus allowing more space for the incorporation of transducing DNA since the $\phi 80$ head specifies the upper limit which can be packaged into a viable phage. The above phages were used as the starting material for this work.

$\lambda\underline{trp}^{+}51$ derivatives carrying the internal deletions trp∇BE9 and trp∇LD102 and the partially characterised deletion/substitution mutation trp∇OE1 were constructed as follows:-

A lysogen of $\lambda\underline{trp}^{+}51$ in W3110 trp∇BE9 was selected as a stable Trp^{+} clone immune to challenge by superinfecting $\lambda\underline{c}$ and $\lambda\underline{h}^{\phi 80}\underline{c}$ as described in Materials and Methods. Induction of this lysogen by ultraviolet light yielded a mixed lysate of the original phage and a derivative able to complement (and hence give Trp^{+} plaques at high efficiency with) W3110trpA33 and A109, but not trpB9700, C9870C10243, D9778 or E9914. $\lambda\underline{trp}^{+}51$ complements all of these mutants and since the former are the properties expected of $\lambda\underline{trp}^{+}51$ carrying the deletion trp∇BE9, one of these phages was repurified by single plaque isolation and the phage stock prepared from it thenceforth referred to as λtrp51∇BE9. Figure 3-1 shows a model for the events involved in its formation.

Since λtrp51∇BE9 lacks trpB,C,D and E function, the phage progeny of any genetic exchange leading to the restoration of any

Figure 3-1 : Proposed mechanism of formation of W3110trp∇BE9(λtrp$^+$51) lysogen and excision of λtrp51∇BE9

λtrp$^+$51 circularises by annealing of its cohesive ends and integrates into the homologous region of the E.coli chromosome. (1) and (2) represent the two recombination events leading to lysogeny. Normal site-specific recombination at the att$^{\phi 80}$ site cannot occur as the int gene is almost certainly deleted in the transducing phage and the normal phage att site is absent. (3) is the reverse excision event; (4) leads to the formation of λtrp51∇BE9. All these events rely on reciprocal recombination and are thus probably mediated by the host rec rather than the phage red system. The prophage arising from event (2) is omitted for simplicity - it leads to an analogous structure with the positions of the trp$^+$ and trp∇BE9 alleles interchanged, and the same excision modes apply.

φ80 DNA is represented by wavy lines, λ DNA by thin straight lines and E.coli by heavy lines.

trpABCDE are the five structural genes of the trp operon, L is the leader region and o/p is the operator-promoter complex.

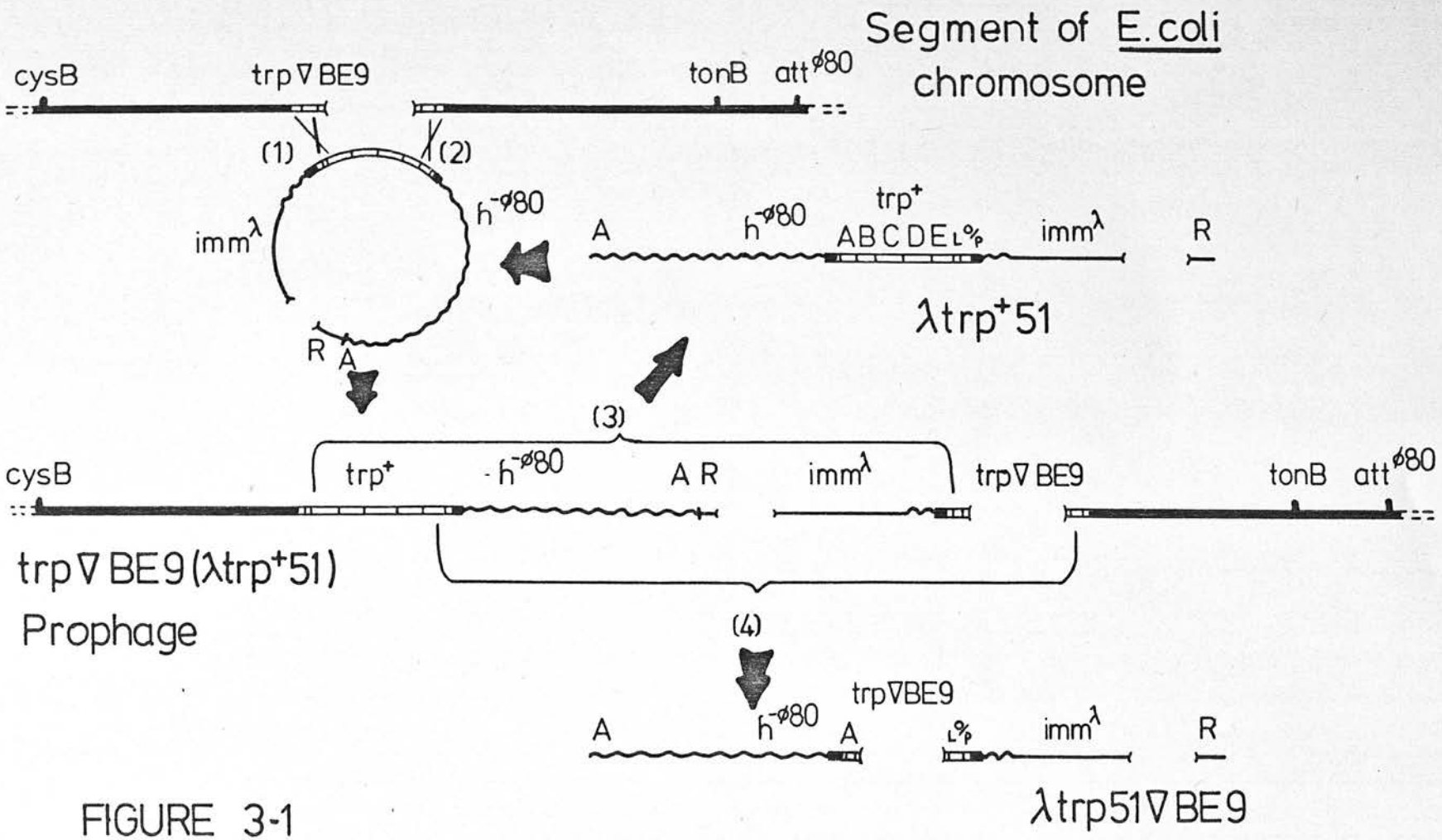

Segment of E.coli chromosome
cysB
trpVBE9
tonB
att$^{\phi 80}$
(1)
(2)
imm$^{\lambda}$
h$^{-\phi 80}$
R
A
trp^{+}
A
h$^{-\phi 80}$
ABCDE
L%
imm$^{\lambda}$
R
λtrp^{+}51
(3)
cysB
trp^{+}
h$^{-\phi 80}$
A R
imm$^{\lambda}$
trpVBE9
tonB
att$^{\phi 80}$
trpVBE9(λtrp^{+}51)
Prophage
(4)
trpVBE9
A
h$^{-\phi 80}$
A
L%
imm$^{\lambda}$
R
λtrp51VBE9

FIGURE 3-1

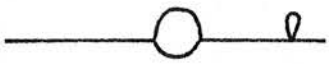
of these functions can be selected directly, even though they may occur at a very low frequency amongst the population as a whole. Such recombinants form Trp^+ plaques on the appropriate (trpB,C,D or E^-) hosts, whereas λtrp51∇BE9 cannot. This situation arises in exchanges with trp∇LD102 and trp∇OE1 : both mutations leave the trpC gene intact and functional, and as both overlap with trp∇BE9 (Figure 1-1), rescue of $trpC^+$ from these mutants must simultaneously result in the pick-up of the respective trp deletions. Employing this strategy, λtrp^+51 derivatives carrying trp∇LD102 and trp∇OE1 were selected from lysates of λtrp51∇BE9 grown on W3110 strains carrying these mutations, as phages able to make Trp^+ plaques on W3110trpC9870C10243. (This double mutant was used because of its very low frequency of reversion to Trp^+.) Further tests showed that the selected recombinants had indeed acquired the complementation patterns expected of ∇LD102 ($TrpA^+B^+C^+D^-E^-$) and ∇OE1 ($TrpA^+B^+C^+D^+E^-$).

λtrp51∇LD102 arose at a frequency of approximately 10^{-3}/plaque forming unit (p.f.u.), but the ∇OE1 derivative was much rarer, occuring at approximately 10^{-5}/pfu. This difference in the frequency of pick-up of these deletions onto the phage suggests that there might be less homology for recombination in the latter case. The host rec system may be responsible for the formation of these phages by a mechanism similar to that outlined in Figure 3-1, here involving only transient lysogeny by integration into and excision from the host chromosome in areas of trp homology, during the normal course of the lytic cycle. Alternatively, the phage red system may perform the conversion;

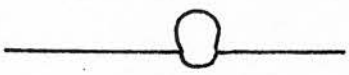
assimilation of an homologous DNA strand from the *trp* region of the host chromosome into the phage DNA could lead directly to the pick-up of the chromosomal marker according to the model postulated by Cassuto and Radding (1971), described in the introducing chapter. Here again, site-specific recombination between phage and bacterial *att* $\phi 80$ sites is precluded because the transducing phages of the λ*trp*51 series all lack the normal phage attachment site and probably also the $\phi 80$ *int* function which catalyses the insertion (lost by substitution of bacterial DNA).

TABLE I

Measurement of heteroduplexes of λtrp51∇ phages

Heteroduplex	Plate Nos	Measurements converted to % λ^+
407.5nin$^+$/λtrp51	9304/5	50.5 — 9.75 — 22.15 — 5.49 — 11.16; 22.15 — 1.59 — 50.5
	9327	50.5 — 10.13 — 24.59 — 5.15 — 9.97; 24.59 — 1.00 — 50.5
	9328	50.5 — 9.12 — 24.49 — 5.40 — 10.81; 24.49 — 1.01 — 50.5
	9329	50.5 — 10.10 — 25.66 — 4.97 — 10.76; 25.66 — 0.99 — 50.5

$\bar{x}$	50.5	9.78	1.15	24.22	5.25	10.68
s.d.	-	0.47	0.30	1.48	0.24	0.50

λtrp51/λtrp$^+$51	9331/2	50.5 — 7.07 — 42.44; 42.44 — 2.47 — 50.5
	9333/4/5	50.5 — 7.50 — 43.00; 43.00 — 2.35 — 50.5
	9336/7	50.5 — 8.71 — 44.58; 44.58 — 2.61 — 50.5

$\bar{x}$	50.5	7.76	2.48	43.34
s.d.	-	0.85	0.13	1.11

$\bar{x}$ is the arithmetic mean of the observations and s.d. the standard deviation about the mean, calculated from the formula $s.d. = \sqrt{\frac{\Sigma(\bar{x}-x)^2}{n}}$, where x is the observed value and n is the number of observations. The actual lengths of the tracings of the heteroduplex were between 130cm and 150cm.

TABLE I (Continued)

Heteroduplex	Plate Nos	Measurements converted to % λ^+
λ*trp*51/ λ*trp*51 ▽102	9342	50.5 — 7.70 — 2.40 — 0.51 — 6.16 — 35.7
	9344/5	50.5 — 7.19 — 2.01 — 0.67 — 5.35 — 34.11
	9397	50.5 — 4.64 — 1.72 — 0.69 — 3.95 — 36.97
	9398/9	50.5 — 3.96 — 1.38 — 0.34 — 4.48 — 34.47

$\bar{x}$	50.5	5.87	1.88	0.55	4.99	35.31
s.d.	-	1.85	0.43	0.16	0.97	1.30

λ*trp*51/ λ*trp*51▽ 0E1	9303	50.5 — 5.95 — 2.16 — 5.41 — 7.21 — 3.79 — 27.05
	9306/8	50.5 — 6.91 — 2.66 — 5.67 — 6.91 — 4.61 — 29.06
	9318/20	50.5 — 7.31 — 2.16 — 5.15 — 8.14 — 4.98 — 28.57
	9324/5	50.5 — 8.04 — 2.27 — 5.94 — 8.56 — 5.24 — 28.31

$\bar{x}$	50.5	7.05	2.31	5.54	7.71	4.66	28.25
s.d.	-	0.87	0.24	0.34	0.77	0.63	0.86

These results are represented diagramatically in Figure 3-2.

Figure 3-2 Heteroduplex mapping of λtrp51∇ phages

Mean double and single strand DNA lengths are given in % λ^+ length. The positions of the trp genes and of the λ site 3 cleaved by EcoRI (srIλ3 : at 65.6% on the standard λ wild type, vegetative map) are shown. The designations trp'C' and trp'E' refer to only part of the genes encoded within the labelled double or single stranded regions of the heteroduplex.

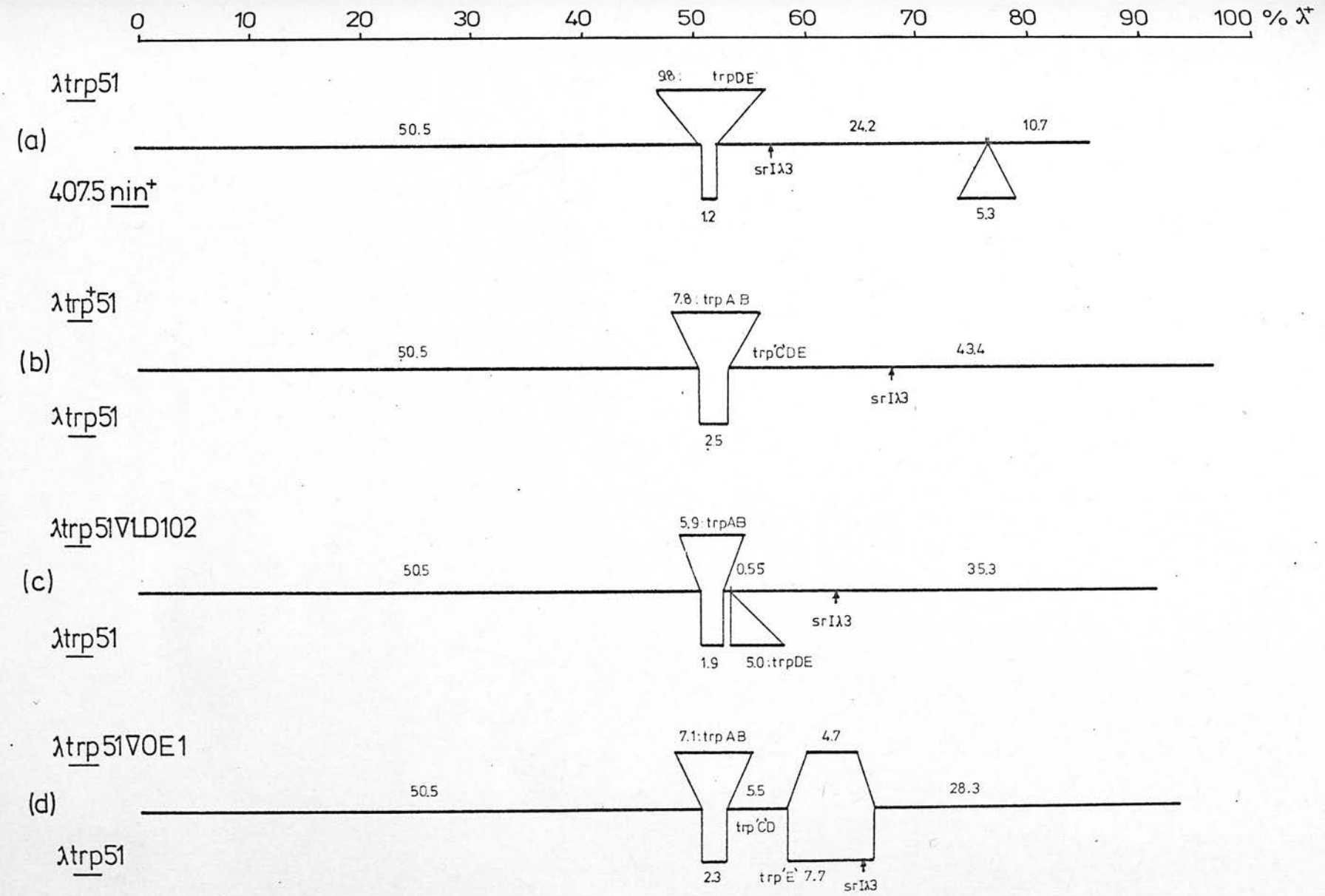


FIGURE 3-2 Heteroduplex mapping of λtrp51∇ phages

(b) Heteroduplex analysis of phages

The phages of the λtrp51 series (λtrp51, λtrp$^+$51, λtrp51▽BE9, λtrp51▽LD102, λtrp51▽0E1 and the λh$^-$ ϕ80att$^{\phi 80}$imm$^{\lambda}$ninR5 hybrid from which they were derived) were characterised further by examination under the electron microscope of DNA heteroduplex molecules formed from selected pairs of phages. Heteroduplexes were prepared as described in Materials and Methods. Photographs were taken of a few examples of each type and these were measured to obtain a low resolution quantitative analysis of the deletion and substitution relationships between pairs of phages.

The results of this survey are presented in Table I and Figure 3-2. The length of the double strand from the left molecular end to the att$^{\phi 80}$ site was taken as the standard and normalised to 50.5% of λ$^+$ double strand length (Fiandt et al. 1971). The formamide spreading technique used yields double stranded and single stranded DNA spread such that the ratio of equivalent double and single stranded lengths is very close to 1.0 (P.J. Highton, personal communication), and this is confirmed by the data shown below. Each of the heteroduplexes will be discussed in turn:

i) Heteroduplex of hybrid 407.5 nin$^+$/λtrp51 (Plate 1)

The nin$^+$ version of the hybrid parent phage of the λtrp51 series (with the ninR5 deletion replaced by λ$^+$ DNA) was used as the nin$^+$/ninR5 non-homology at the conventional 'right' end of heteroduplex molecules allows assignment of orientation by

Schematic representations:

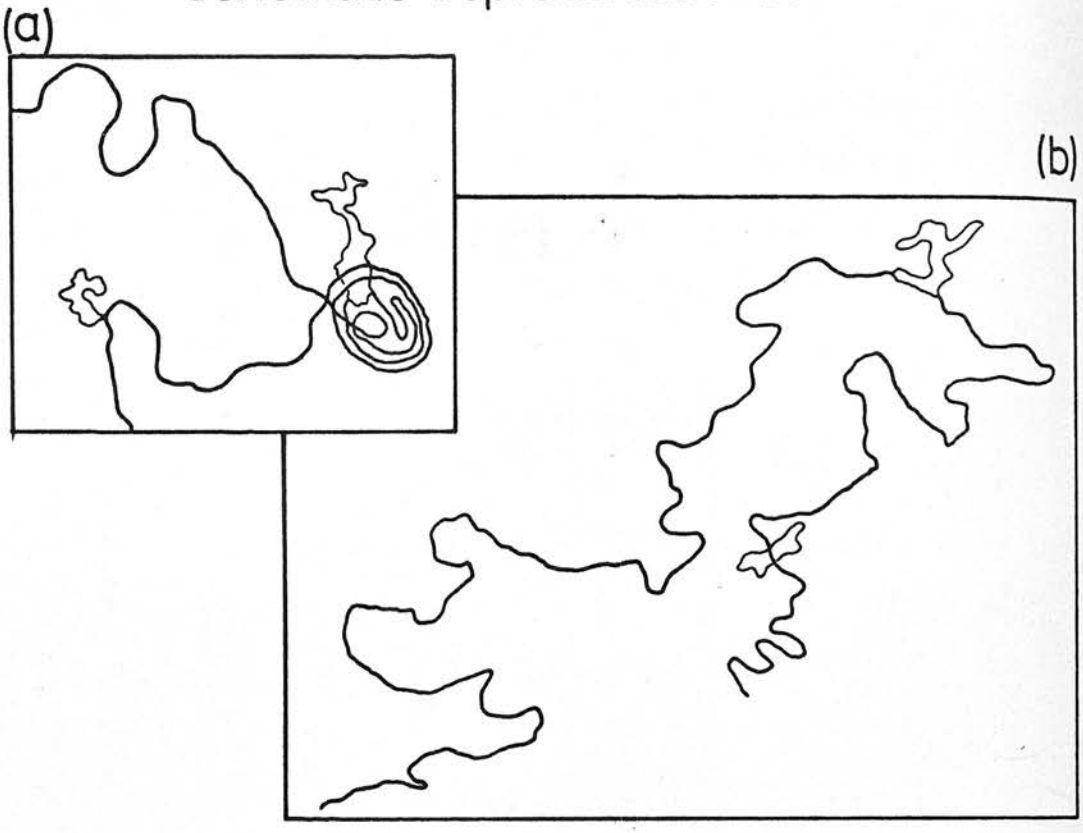


Plate 1 : 407.5 nin^{+} / λtrp51

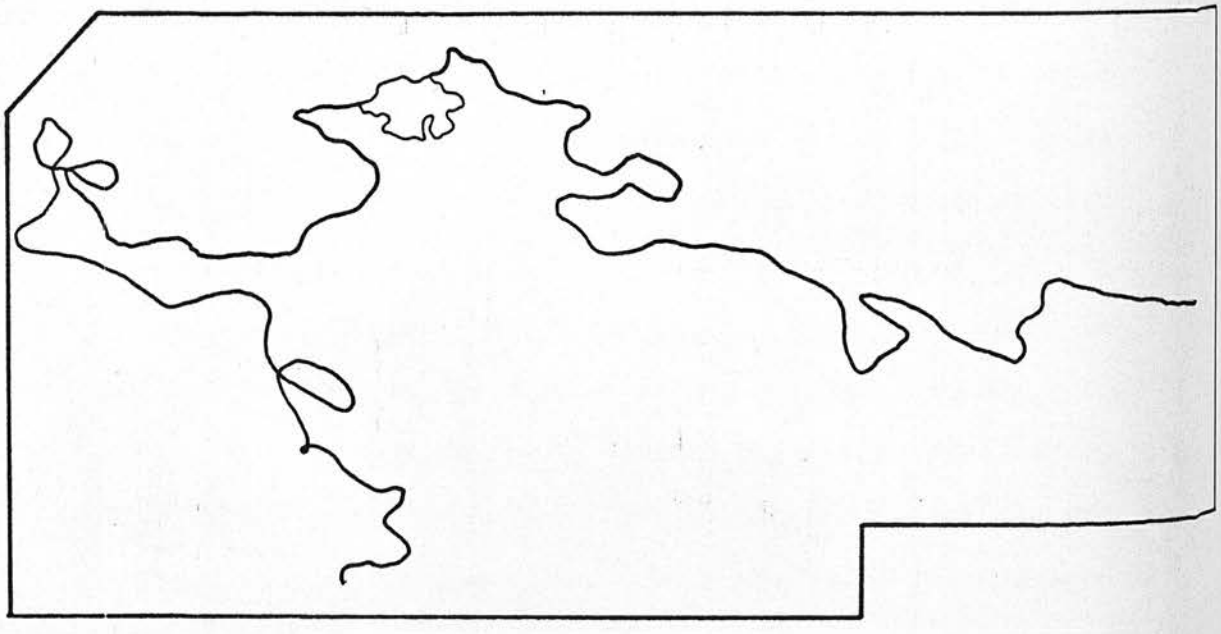


Plate 2 : λtrp51 / λtrp^{+}51

(a)

(b)

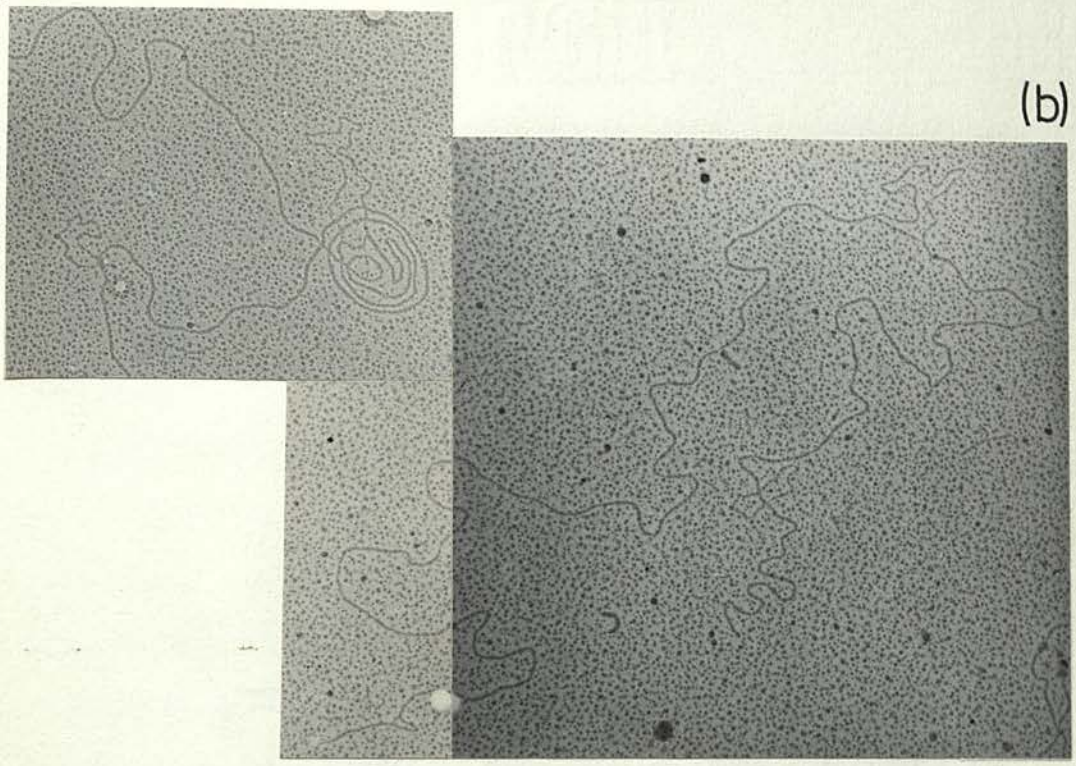


Plate 1 : 407.5 *nin*$^{+}$ / λ *trp*51

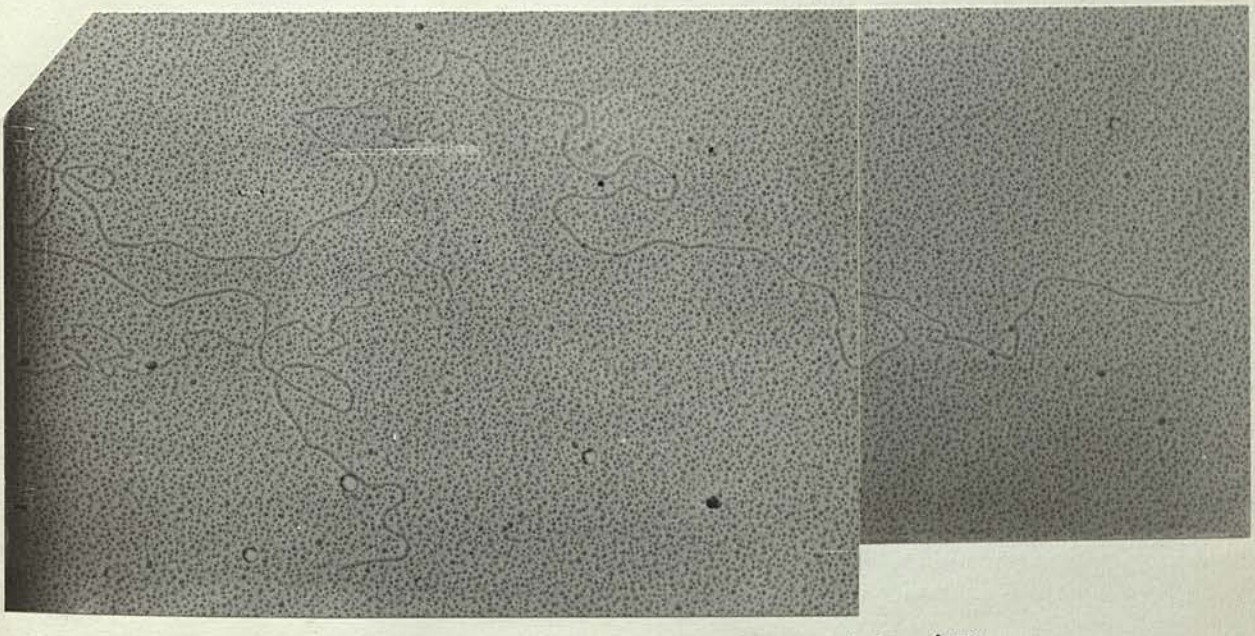


Plate 2 : λ *trp*51 / λ *trp*$^{+}$51

inspection, and also provides a useful reference loop of known size and position as a check on the accuracy of these measurements. This *nin*$^+$ hybrid was annealed with λ*trp*51 and showed the expected DNA heteroduplex of a single small deletion loop near one end and a substitution bubble near the middle of the molecule. The deletion is *nin*R5 and the substitution is the replacement of φ80 DNA between *att* and *xis* by bacterial DNA containing the *trp* D and E genes.

The compiled results, shown in Table I, are in good agreement with the known characteristics of *nin*R5; 5.3% λ$^+$ deleted at a position 10.7% from the right molecular end (as compared with the accepted figures of 5.4% and 10.8% respectively, Fiandt *et al.* 1971). Thus the use of the φ80 left arm as the internal standard is vindicated. The lengths of the single stranded arms of the substitution bubble are 1.2% and 9.8% λ$^+$ length, making the assignment of strands unambiguous. The 1.2% (590 base pair) arm must correspond to φ80 DNA replaced by the 9.8% (4,800 base pair) length of bacterial DNA, since the former is insufficient to encode the *trp* D and E genes (each with molecular weights of 60,000 daltons, requiring a minimum of 3,300 base pairs for the combined length of their genes), and the latter is too large to be accommodated between *att* and *xis* of φ80. (Figure 3-2(a).)

ii) *Heteroduplex of λtrp51/λtrp$^+$51* (Plate 2)

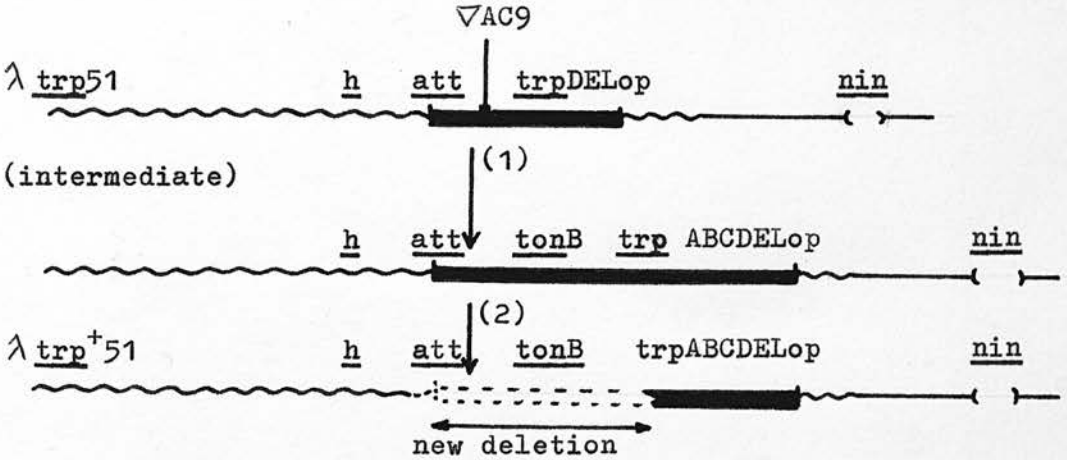
The relationship between the (*ton*B-trpABC)∇ trpD$^+$E$^+$ transducing phage, λ*trp*51, and its *trp*$^+$ derivative was examined directly in

this heteroduplex which was shown to carry a single substitution bubble near the middle of the molecule. By comparison with the previous results, the longer double stranded arm must correspond to the conventional left side of the molecule, and this was normalised to 50.5% λ^+ length as before, (Table I). The alternative choice yields much larger values for the total length of DNA of both phages (in excess of 110% λ^+) which do not concur with that previously observed for λ trp51, whereas the present results are in good agreement with the data of figure 3-2(a).

The 7.8% λ^+ length (3,800 base pair) substitution arm must be the acquired DNA including the trp A and B genes, and the 2.5% λ^+ length (1,200 base pair) arm must represent DNA lost in the exchange. (Figure 3-2(b).) This is the only possible assignment as the trp A and B proteins, of molecular weights 29,500 and 50,000 daltons respectively, require a total of approximately 2,200 base pairs to encode their genes.

The fact that the heteroduplex shows a substitution bubble rather than a deletion loop is surprising at first sight, since the chromosomal marker ∇ AC9 carried by λtrp51 is a pure deletion of the tonB-trp region of the host chromosome (Yanofsky et al., 1971; Coukell and Yanofsky, 1971). However, several lines of evidence support the contention that λ trp$^+$51 was formed from λ trp51 by two independent events, which when taken together resemble a substitution. First, the trp$^+$ phage arose at an abnormally low frequency from a lysate of λ trp51 on a trp$^+$ host (W.J. Brammar, personal communication). Second, in previous

studies the major class of φ80p*trp* transducing phages obtained by induction of a φ80$^+$ lysogen carried only part of the *trp* operon (Matsushiro, 1963; Deeb, Okamoto and Hall, 1967). The only *trp*$^+$ transducing phage of this type (φ80pt190*h*) was obtained as a rare variant amongst a lysate induced from a T1-resistant (*tonB* ∇) host lysogenic for φ80*h*, indicating that there is some obstacle to the direct formation of such *trp*$^+$ transducing phages. Thirdly, several *h*$^-$ φ80p*trp* transducing phages isolated from *ton*$^+$ hosts do not transduce the T1-receptor, *tonB*, (W.J. Brammar, personal communication) which maps between the attachment site for φ80 and the *trp* operon, and should therefore be carried by *trp* - transducing phages generated by a simple aberrant cross-over mechanism. (See also the test for *tonB* transduction by λ*trp*$^+$51, later in this chapter) These observations suggest a model for the events giving rise to φ80p*trp* transducing phages, detailed below for the genesis of λ*trp*$^+$51.



Schematic representations:

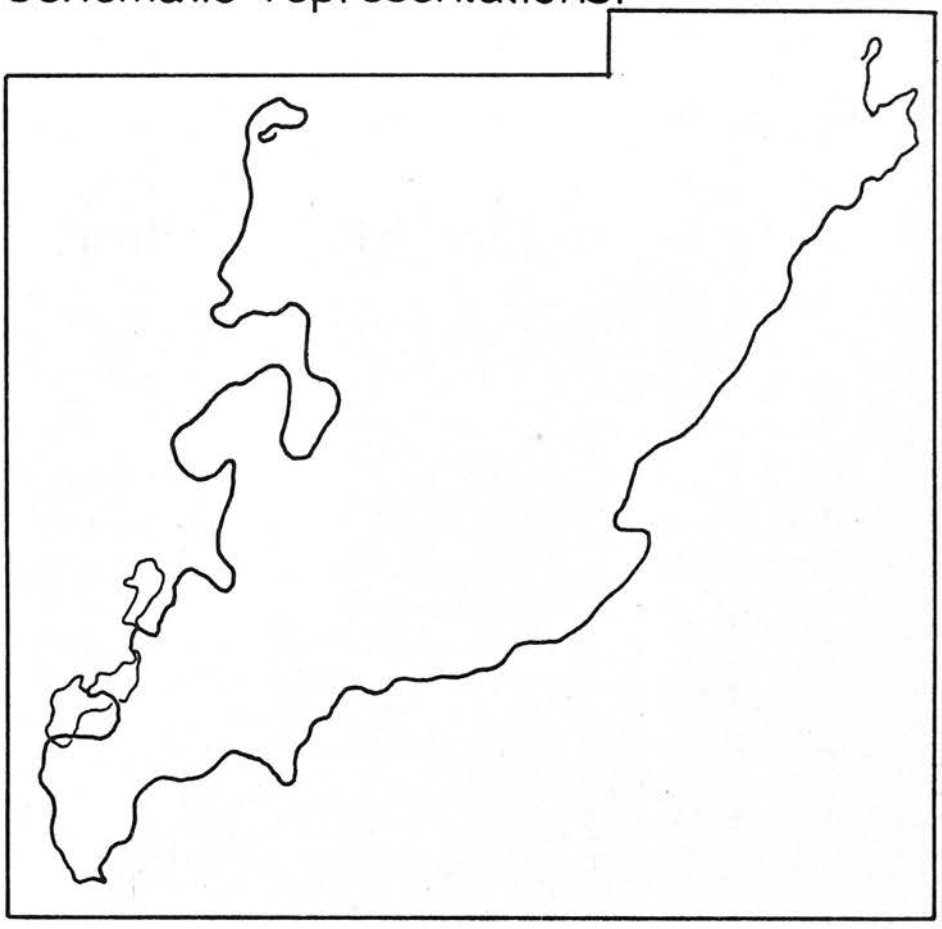


Plate 3 : λtrp51 / λtrp51∇LD102

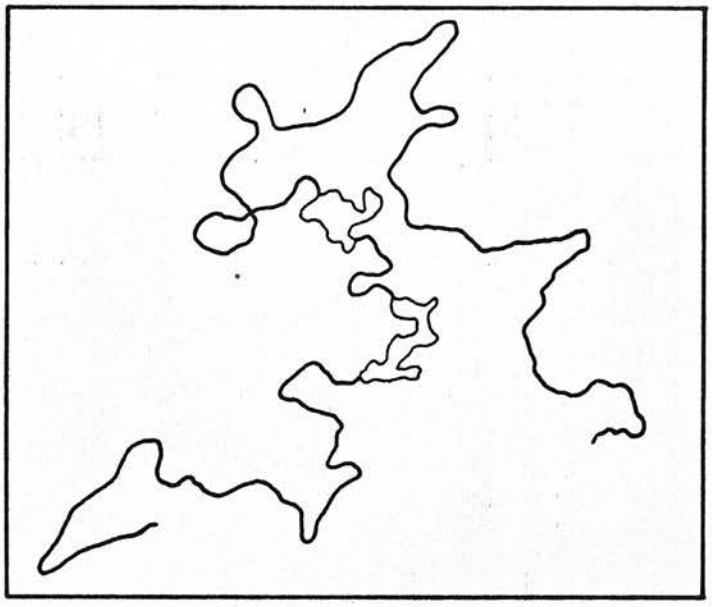


Plate 4 : λtrp51 / λtrp51∇OE1

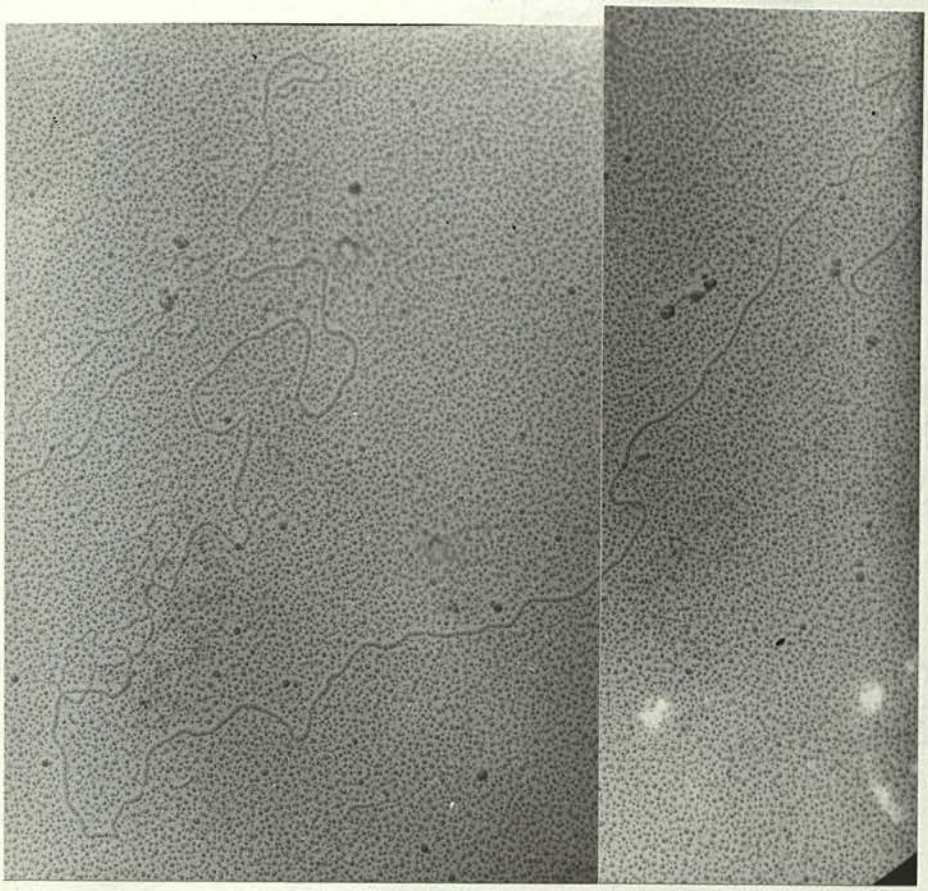


Plate 3 : λtrp51 / λtrp51∇LD102

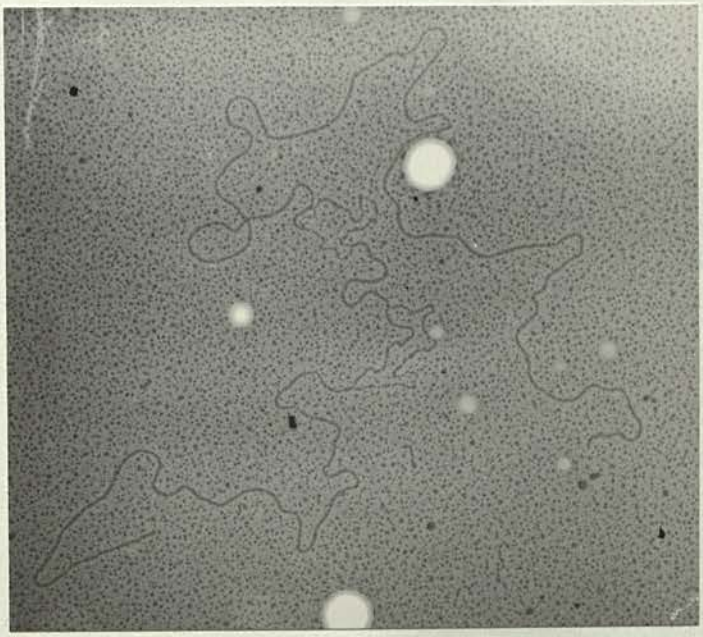


Plate 4 : λtrp51 / λtrp51∇OE1

Event (1) may require host rec or phage red functions, yielding a phage which replicates normally, but is in this instance far too large to be packaged into the ϕ80 head.

Event (2): A very strong selection now pertains for derivatives which have deleted sufficient DNA to bring the size of the remaining transducing phage DNA to within the packageable limits whilst retaining the trp operon intact. This constraint demands extensive deletions of inessential DNA, and those between the ϕ80h gene and trp may well be the only ones which satisfy these criteria.

According to this model λtrp$^+$51 has gained 5.3% (7.8 - 2.5) λ^+ length of DNA from the promoter distal CBA end of the trp operon, and deleted a further 2.5% λ^+ length of the original λtrp51 phage DNA from the end of the deletion AC9 towards or across the phage att site. This in turn means that the use of the ϕ80 left arm as a standard normalised to 50.5% λ^+ could be in error by up to 2.5% λ^+ length, but in fact the total length of λtrp51 calculated on this basis is 96.3% λ^+ length as compared with 95.2% from the heteroduplex (a) in Figure 3-2. This, within the limits of resolution of the present data, any loss of ϕ80 DNA left of the att site in λtrp$^+$51 must be very small.

iii) Heteroduplex of λtrp51/ λtrp51∇102 (Plate 3)

Since λtrp51∇LD102 was derived from λtrp$^+$51 (via λtrp51∇BE9), the substitution non-homology between λtrp51 and λtrp$^+$51 would be anticipated in this heteroduplex in addition to

the deletion loop from ∇LD102. This structure, a substitution bubble near the middle of the molecule very close to a deletion loop, is seen as predicted. The left arm (identified as that on the opposite side of the deletion loop) was again normalised to 50.5% λ^+ length, and this is subject to the same small systematic error discussed above.

These data (Table I) lead to an estimate of 5% λ^+ length (2,500 base pairs, with a standard deviation of 500 base pairs) for the length of deletion LD102; this is lower than the 3,400 base pair estimate obtained from genetic data (Jackson & Yanofsky, 1972; Figure 1-1). The small double stranded DNA link between the substitution and deletion, defining the distance between the endpoints of deletions AC9 and LD102, was measured as 0.55% λ^+ length, corresponding to 270 base pairs with a standard deviation of 80 base pairs. This too is a lower estimate than that obtained from genetic data (Yanofsky et al. 1971), which suggests a value of approximately 600 base pairs for this distance. The overall length of λtrp51 assessed from these heteroduplexes is 93.2% λ^+, some 2-3% lower than the above estimates, but not significantly at variance with them.

iv) Heteroduplex of λtrp51/λtrp51∇OE1 (Plate 4)

In this heteroduplex the λtrp51/λtrp$^+$51 substitution was again anticipated along with the OE1 non-homology of an unpredicted nature. The heteroduplexes observed, Table I, shows two substitution bubbles. One, near the middle of the molecule

must be the λ trp51/λ trp$^+$51 non-homology referred to above, and is of the same dimensions as those seen previously (compare (b) and (d) in Figure 3-2), and the other, with single stranded arms of more nearly equal length, must be the change due to OE1.

To decide which of the arms is the new DNA strand incorporated into λ trp51 ▽ OE1 and which is the replaced strand, the figure of 94.3% λ$^+$ length was used for the total length of phage λ trp51 DNA. (This is the average of the values obtained from the previous three heteroduplexes from Figure 3-2 (a), (b) and (c).) The longer of the two strands (7.7% λ$^+$ length) was assigned as the λ trp51 DNA replaced by the shorter (4.7% λ$^+$) length of new DNA, since this leads to an estimate of 94.3% (50.5+2.3+5.5+7.7+28.3)% λ$^+$ length for the total phage DNA, whereas the alternative arrangement would lead to a value of 91.3% (50.5+2.3+5.5+4.7+28.3): Values taken from Table I. This is a narrow margin for the decision, but the unique predictions of this alternative, that the site for the restriction enzyme EcoRI in λ red (at a position of 65.6% on the standard λ$^+$ map, Thomas & Davis, 1975) and at least part of the λ red α,β region are removed by the OE1 substitution in λ trp51 ▽ OE1 (Figure 3-2 (d).) are amply confirmed, as described later in this chapter.

v) Heteroduplex of λ trp51 ▽ OE1/ λ trp51 ▽ BE9 (Plate 5)

Heteroduplex molecules between λ trp51 ▽ OE1 and λ trp51 ▽ BE9 were prepared and examined under the electron microscope, but were not measured, since only the position of one end of the BE9

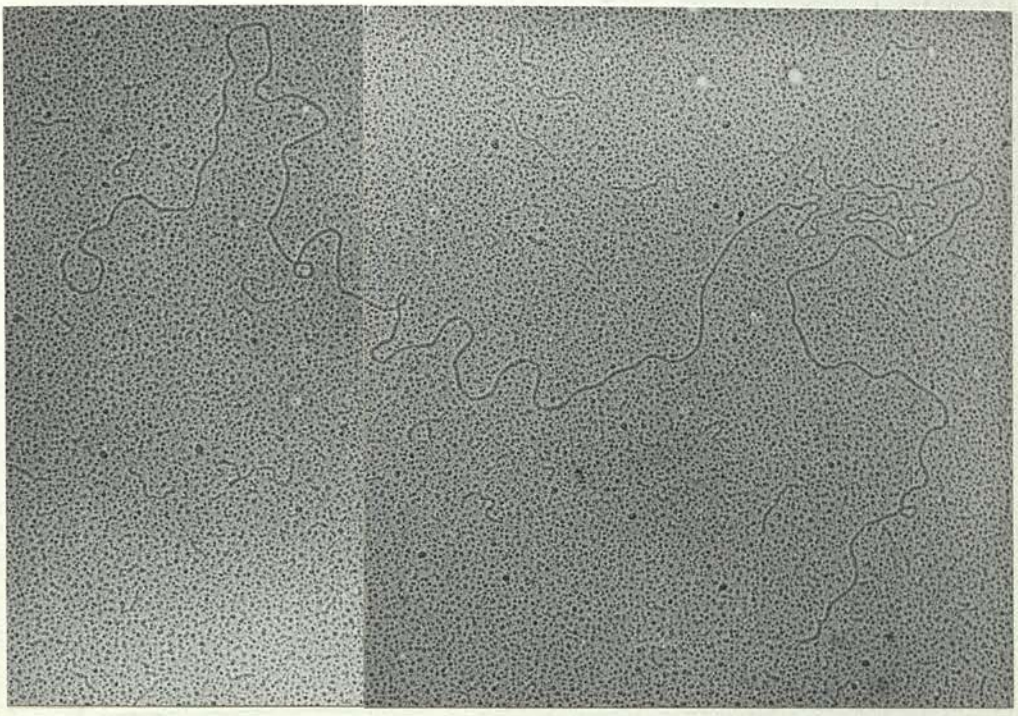
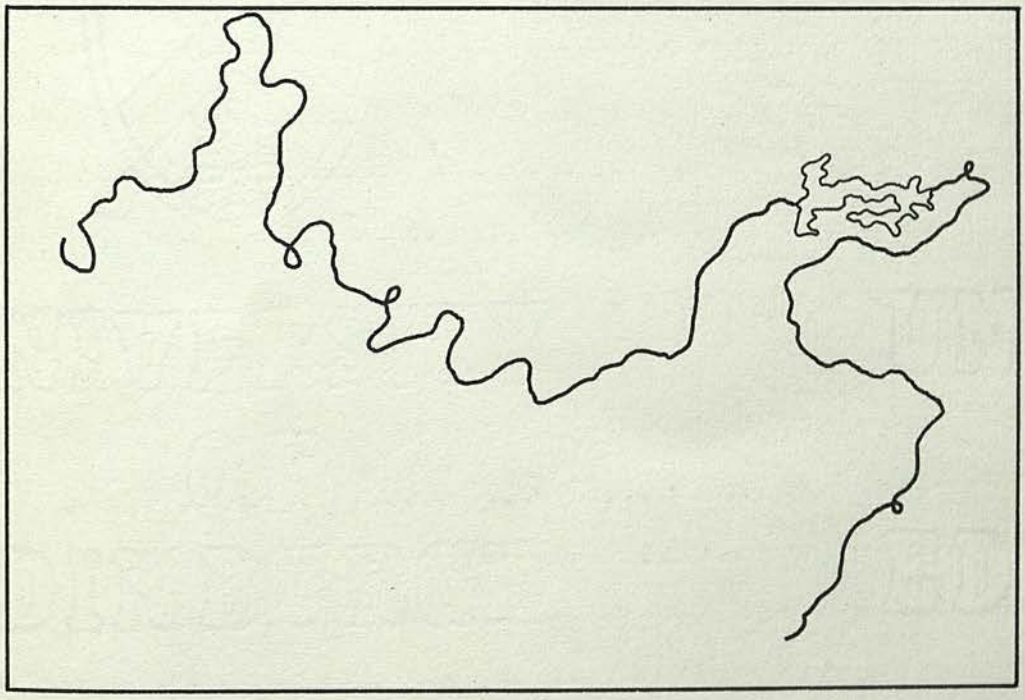


Plate 5 : λtrp51∇0E1 / λtrp51∇BE9



deletion would be determined (the other being under the OE1 replacement), and these results would only partially characterise λ trp51∇BE9. The heteroduplex formed between λ $\underline{trp}^{+}$51 and λ$\underline{trp}$51∇BE9 would isolate the $\underline{trp}^{+}$/∇BE9 non-homology, but would carry no standard length of double stranded DNA and so was not examined.

The heteroduplex formed (Plate 5), showed the expected pattern of a large central substitution bubble, which can be explained by an overlapping deletion and deletion/substitution, as predicted for BE9/OE1 in this case.

(c) Further genetic analysis of λtrp51 series

i) TonB tests

From the considerations of the mechanism of formation of λtrp$^+$51 from λtrp51 in the previous section, it is of interest to know whether the trp$^+$ phage carries the tonB locus intact. Since the latter lies between att$^{\phi 80}$ and trp on the E.coli chromosome, it would be expected to be present in a λh$^{\phi 80}$trp transducing phage ~~(phage)~~ formed by the simple single step Campbell model of abnormal excision of a prophage (Campbell, 1957). Such a λh$^{\phi 80}$tonBtrp transducing phage should express tonB in the prophage state.

A lysogen of λtrp$^+$51 in a host carrying a tonB deletion was tested for its TonB phenotype. A W3110(tonB-trp)∇AE8(λtrp$^+$51) lysogen was constructed as described in materials and methods, as a stable Trp$^+$ clone immune to challenge by λc and λh$^{\phi 80}$c. Integration, a prerequisite for stable lysogeny, can only occur by rec mediated recombination within the limited homology remaining between the ∇AE8 region of the host and the transducing DNA of the phage, as in Figure 3-1. φ80vir was unable to form plaques on this lysogen (efficiency of plating, eop $< 10^{-6}$), whereas φ80h$^-$ grew well (eop approximately 1.0), showing that the growth of φ80 was not blocked by the prophage, nor by any host defect other than the phage receptor deficiency. These are the properties of the parent tonB host, and so the conclusion was drawn that λtrp$^+$51 does not carry tonB intact.

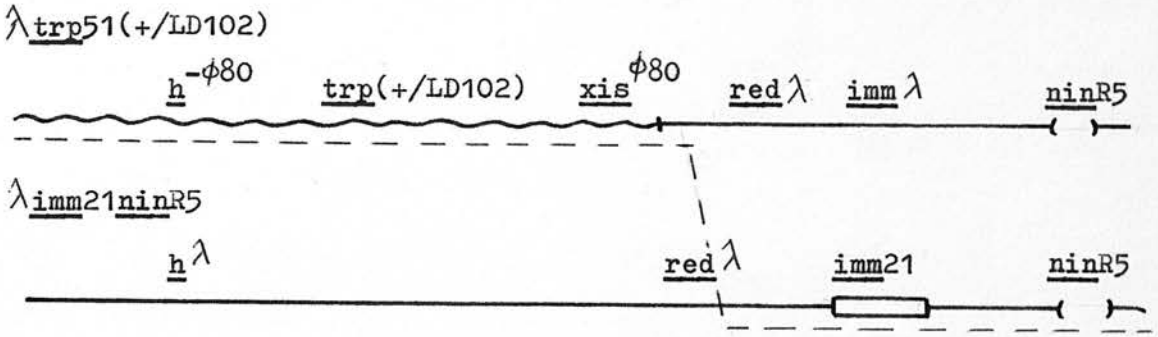
ii) Tests for the K-restriction site of $\phi 80$

Phage 407.5, the λ: $\phi 80$ hybrid progenitor of the λ *trp*51 series, has the left arm of its genome contributed by $\phi 80$ and the right arm by λ (see the beginning of this chapter). It has been shown to carry *xis* $^{\phi 80}$ and also the site for the *Eco*R1 enzyme in λ *red* α ($\phi 80$ DNA has no analogous site; the nearest being those left of *att* $^{\phi 80}$ and that within the immunity region of $\phi 80$: Helling Goodman & Boyer, 1974): Figure 1-2 . The position of the cross-over from $\phi 80$ to λ DNA in this phage is thus not accurately known.

The single site in $\phi 80$ DNA for the restriction enzyme *Eco*RK (*sk*80) resides in this region of the $\phi 80$ genome. Murray, Manduca de Ritis & Foster (1973) showed that its position is between the end of the $\phi 80$ region equivalent to deletion *b*522 and $\phi 80$ *red*, placing it between 64.3% and 65.6% on the homologous map of λ DNA: Figure 1-2 .

The experiments described below were designed to test whether λ *trp*51 and hybrid 407.5 carry *sk*80, so as to give more precise information concerning the position of the $\phi 80$:λ DNA exchange. In order to detect *sk*80 by the twenty fold increase in K-restriction it confers on an unmodified phage, no more than one other K site should be present, any additional sites it contains must be replaced or deleted. The hybrid 407.5 should also contain *sk* λ1 and *sk* λ2 and λ*trp*51 should have *sk* λ1, *sk* λ2 and the K restriction target in trp E, *sktrp* (Murray & Brammar, 1973), as well as *sk*80, if the latter is present.

The replacement of the immunity region of λ by that of phage 21 is known to remove sk λ2 without introducing any other K recognition sites, and the internal trp deletion LD102 removes sktrp along with the entire trpE gene sequence. The imm21 derivatives of λ trp$^+$51 and λ trp51 ∇ LD102 were constructed as follows:



The hybrid trp transducing phages were crossed to λ imm21 ninR5 in the host strain W1485 as described in Materials and Methods, selecting h φ80 imm21 recombinants on W1485(λ)/λ. λ trp$^+$51 imm21 and λ trp51 ∇ LD102 imm21 recombinants were purified by single plaque isolation on W1485/λ and shown to carry imm21 by failure to grow on W1485(λ imm21).

Non-modified stocks of these two phages and also of λ cI26, φ80$^+$, hybrid 407.5 and λ trp51 ∇ LD102 were prepared on E.coli strain C-1a. Tenfold serial dilutions of these were spotted onto BBL plates overlaid with soft agar seeded with either the K-restricting strain W1485 or the non-restricting strain C-1a, in order to determine their K-restriction ratios, Table II(a).

TABLE II

K-Restriction Ratios of λtrp51 Series

(a)

Phage	K restriction sites						K restriction * ratio
	sk80	skλ1	skλ2	Other skλ	sktrp	Σ sites	(titre on Cla) / (titre on W1485)
λcI26	-	+	+	+++	-	3	2,500
φ80+	+	-	-	-	-	1	10
hybrid 407.5	?	+	+	-	-	2 or 3	3,000
λtrp+51 imm21	?	+	-	-	+	2 or 3	5,000
λtrp51▽ LD102	?	+	+	-	-	2 or 3	250
λtrp51▽ LD102 imm21	?	+	-	-	-	1 or 2	100

TABLE II (Continued)

(b)

Phage	K restriction sites					K restriction* ratio
	sk80	sk λ1	sk λ2	Other skλ...	Σ sites	
λ cI26	-	+	+	+++	3	2,000
φ80+	+	-	-	-	1	15
hybrid 407.5 'flip' (i)	?	-	+	-	1 or 2	500
hybrid 407.5 'flip' (ii)	?	-	+	-	1 or 2	1,000
λ trp51 LD102 imm21 'flip' (i)	?	-	-	-	0 or 1	16
λ trp51 LD102 imm21 'flip' (ii)	?	-	-	-	0 or 1	18

* The restriction coefficient of a phage is defined as the titre of unmodified phage on a non-restricting host divided by that on a K-restricting host.

The results show λ<u>c</u>I26 and φ80$^+$ have K restriction ratios of 2,500 and 10 respectively. These figures are 2-3 fold lower than those usually observed, but since titres were estimated by spotting dilutions (a procedure which is only reliable to within 2 fold), they are quite consistent with those expected. On this basis, hybrid 407.5 and λ<u>trp</u>$^+$51<u>imm</u>21 show ratios of 3,000 and 5,000 respectively, indicative of three or more sites each; λ<u>trp</u>51: ∇ LD102, 250, consistent with two or possibly three sites in view of the generally low ratios observed; and λ<u>trp</u>51∇LD102<u>imm</u>21 shows a ratio of 100, which is suggestive of two sites, since 50 is the highest value observed for a single site, and hence provides evidence that <u>sk</u>80 is present in these phages.

At the suggestion of Dr N.E. Murray, <u>sk</u>λ1 in these phages was removed by replacement of the 'right tip' of λ DNA (on the conventional vegetative phage map) by the analogous φ80 DNA (which is known to be devoid of recognition sites for the K restriction enzyme) by growth on a φ80 lysogen - this process almost invariably leads to replacement of <u>sk</u>λ1 (N.E. Murray, Manduca de Ritis & Foster, 1973). Such 'flipped' <u>h</u>$^{\phi 80}$<u>imm</u>λ hybrids are recognised by their high efficiency of plating (eop = 1.0) on a φ80 lysogen, as compared with that of the original hybrid with λ DNA at the right tip, which was 20-30 fold lower.

λ<u>trp</u>51∇LD102<u>imm</u>21 with the φ80 right tip removing <u>sk</u>λ1 should thus retain only the putative <u>sk</u>80. This hypothesis was tested as follows: hybrid 407.5 and λ<u>trp</u>51∇LD102<u>imm</u>21 were grown

on W1485(ϕ 80) and each shown to plate with low efficiency (eop = 0.03). Two isolates of each phage were selected. Subsequent growth of these phages on the same host was with the usual high efficiency (eop = 1.0). Unmodified stocks of these phages were prepared on E.coli C1a, and their K-restriction ratios determined by spot titration on W1485 and C1a as before. Table II(b).

The control phages λ cI26.c and $\phi 80^{+}$.c gave ratios of 2,000 and 12 respectively (again approximately two fold lower than normally seen with accurate full plate titrations) and those of the two hybrid 407.5 'flips' were 1,000 and 500 (characteristic of at least two sites) whilst the λtrp51∇LD102imm21 'flips' were still restricted 16 and 18 fold, indicating the presence of a single K restriction target. The latter two phages thus retain sk80 which must also be present in all the λ trp51 series (with the notable exception of λtrp51∇OE1: see the next section) which retain the ϕ80 DNA between xis and red.

The cross-over from ϕ80 to λ material in these phages must therefore be right of the ϕ80DNA equivalent to deletion b522, and left of λ red.

iii) Tests for red and gam functions in phage λtrp51∇OE1

One of the predictions of the λtrp51/λtrp51∇OE1 heteroduplex data is that at least part of the redα-redβ-gam region is removed by the OE1 substitution in phage λtrp51∇OE1. Several genetic properties conferred by this region of the genome can be conveniently tested: the Spi, Fec and Feb phenotypes (See introductory chapter). Results of these tests with λtrp51∇OE1 and control phages are shown below:

Growth of phages on various hosts

Phenotype: / phage: / host:	Spi 594(P2)	Fec QR48(recA)	Feb N3098(ligts7)
λ^+	-	+	+
λpbio-1	+	-	-
λtrp$^+$51	-	+	+
λtrp51∇OE1	-	+	-

λpbio-1 removes both red and gam by substitution, and thus shows the Spi$^-$,Fec$^-$,Feb$^-$ phenotypes. λtrp$^+$51, which carries red and gam intact, (Brammar et al. 1974) behaves as does λ^+ (Spi$^+$,Fec$^+$,Feb$^+$) whereas the OE1 derivative is Spi$^+$ and Fec$^+$ but Feb$^-$, indicating only a lesion in either the red genes or gam. Since the OE1 replacement in this phage approaches the red-gam region from the left, red would be expected to be affected leaving the gam gene intact.

(d) Analysis of trp operon DNA in $\lambda trp^{+}51$ using EcoRI and Hind II & III

The effects of substitutions, additions and deletions on the restriction fragment pattern of a DNA molecule are discussed in Appendix I.

It is clear that the simplest situation is found in cases where the region affected by the change is bounded by a pair of cleavage sites. In such cases, provided no new sites are introduced, the two related molecules yield restriction digests differing in only one fragment. EcoRI makes only five breaks in a wild type Lambda DNA molecule (Allet et al., 1973; Murray & Murray, 1974; Thomas & Davis, 1975) and was expected to produce a simple restriction fragment pattern with the DNA of the $\lambda trp51$ series of phages. A more detailed picture of the changes can be obtained by analysing digests of these phage DNAs with a restriction enzyme which makes many cuts per molecule. The unfractionated mixture of the two restriction endonucleases Hind II & III, which introduces forty-three breaks in the Lambda genome (Allet & Bukhari, 1975), was used to derive a fine structure map of the trp operon DNA in $\lambda trp^{+}51$ and its derivatives.

(i) Analysis with EcoRI: Evidence presented earlier in this chapter shows that the trp operon in $\lambda trp^{+}51$ replaces $\phi 80^{+}$ DNA of the att-xis region of the hybrid phage from which it was derived, and that this region is known to be devoid of targets for the EcoRI enzyme. Also, since the trp operon DNA itself lacks such

targets (Brammar, Murray and Winton, 1974) deletions, substitutions or any other structural changes which lie within the trp DNA should affect the length of only one fragment of an EcoRI digest. That this situation prevails for all the λtrp51 series of phages, with the sole exception of λtrp51∇OE1, was demonstrated by analysis of EcoRI digests.

Plate 6 shows the resolved EcoRI fragments of the DNA of the λtrp51 series of phages (tracks 2 - 7), along with those of $\phi 80^+$ (track 8), wild type λ (tracks 1 & 9) for calibration purposes and λ trp^+51hλ (track 10) to help in the assignment of φ80 derived fragments. The λtrp51 series show nine bands in common and these were designated a - i, whilst the λ:EcoRI fragments were designated A - F, as shown in Plate 6.

The λtrp51 series each show one unique band (λtrp51∇OE1 also lacks band b) as predicted. This must in each case contain the trp DNA, or the φ80 DNA for which it is substituted in the case of the parental hybrid phage, 407.5 : track 6. Bands B and b, as well as F and f are equivalent (Figure 1-2) and are derived from the immunity region and the extreme right tip of λ, respectively. Bands c,d,e,h and i are all present in the φ80 DNA digest (track 8) and thus originate from the left arm of the hybrid phages.

Bands a and g remain to be assigned. It is known that the λ trp phages also contain a fragment derived from λ:EcoRI C containing the ninR5 deletion, and thus yield a fragment (12.1-5.4) 6.7% λ^+ length, smaller than λ:EcoRI F of 6.9% λ^+

Plate 6 EcoRI digests of λtrp phage DNA

1.0 ug of each phage DNA was digested with EcoRI under the standard conditions described in Chapter 2 (t). Digests were submitted to electrophoresis through a 1% agarose slab gel, stained and visualised as detailed in Chapter 2 (u). The origin is at 40 cm on the scale. The DNA species were:

Track
1. λ^+
2. λ*trp*51 ∇ OE1
3. λ*trp*51 ∇ BE9
4. λ*trp*51
5. λ*trp*51 ∇ LD102
6. $\lambda h^{-}\text{ø80}\,nin\text{R5}$ (hybrid NM407.5)
7. $\lambda trp^{+}51$
8. ø80^{+}
9. λ^+
10. $\lambda\, trp^{+}51h^{\lambda}$

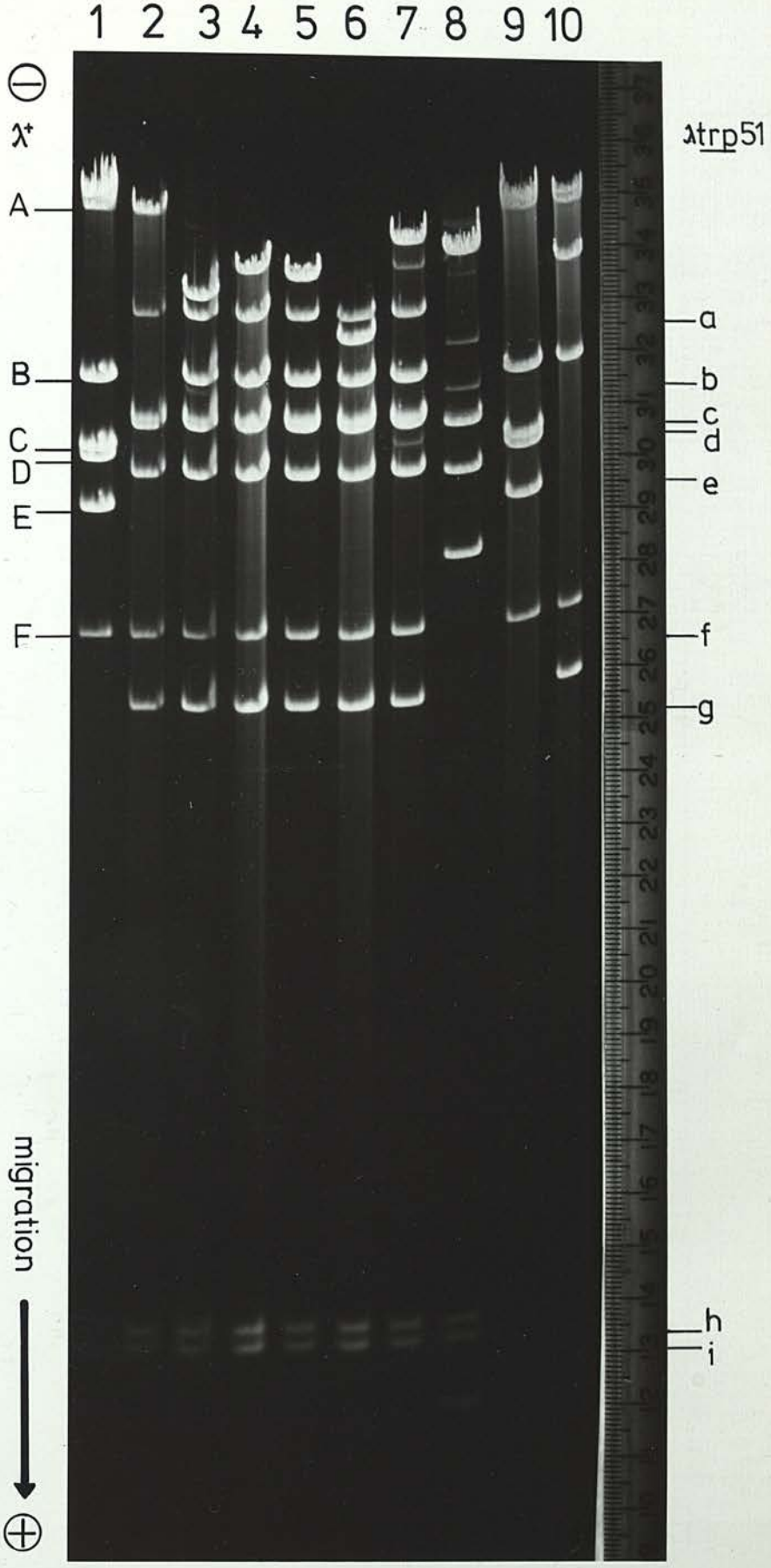


Plate 6

length. This must be represented by band g, though measurements from Plate 6 suggest that ninR5 extends over 6.0% rather than 5.4% λ^+. Band a appears to be derived from neither λ^+ nor $\phi 80^+$ and cannot arise from the region of their exchange in the hybrids, as this has been shown to be within the trp : EcoRI fragment. Annealing of the terminal fragments c and f, however, would produce a large fragment with a calculated mobility similar to that observed for band a (See Appendix II). This interpretation has not been tested further. Track 10 shows a digest of the hλ derivative of λtrp$^+$51, which replaces most of the $\phi 80$ left arm of the hybrid transducing phage by λ material, and as might be anticipated, this removes bands a,c,d,e,h & i.

The known molecular weights of the λ : EcoRI fragments were plotted against the observed mobilities of their bands as observed from Plate 6, and a calibration curve of molecular weight vs. mobility obtained (Appendix II). This was used to estimate the molecular sizes of the unique EcoRI fragments of the λtrp51 series giving the following results:

Phage DNA and EcoRI fragment	Molecular Size		
	Md*	Kb*	% λ^+ *
407.5 (att-xis) fragment	6.0±0.4	9.0±0.6	18.4±1.2
λ trp51 : trpAC9 fragment	9.1±0.6	13.6±0.9	27.8±1.8
λ trp$^+$51 : trp$^+$ fragment	11.3±0.9	17.0±1.4	34.5±2.8
λ trp51∇ BE9 : trp fragment	7.8±0.5	11.7±0.8	23.8±1.6
λ trp51∇ LD102 : trp fragment	8.9±0.6	13.4±0.9	27.2±1.8
λ trp51∇ OE1 : trp fragment	13.5±1.5	20.2±2.2	41.2±4.4

* Units quoted are Md (mega-daltons : daltons $x10^{-6}$), Kb (Kilo-base pairs : base pairs $x10^{-3}$) and % λ^+ length (taking the estimate of 49Kb as 100% λ^+ length). The latter were included to facilitate comparison with the heteroduplex results described earlier. Errors were estimated on the basis of an uncertainty of 2mm in the band position.

Only BE9 and LD102 are represented as pure deletions in their respective λ *trp*51 derivatives, and the above data lead to estimates of 5.3Kb and 3.6Kb for the extent of ∇ BE9 and ∇ LD102, respectively, by comparison with λ *trp*$^+$51. Deletions AC9 and OE1 have been introduced concomitantly with substitutions, the net size reductions being 3.4Kb and 4.15Kb, respectively, by comparison with λ *trp*$^+$51, (the ∇ OE1 derivative having absorbed λ:*Eco*RI fragment B into the *trp* fragment present in its digest). Similarly the substitution of the *trp*DE genes for the *att*-*xis* region of ϕ80 in the formation of λ *trp*51 from the hybrid, 407.5, led to an estimated net increase of 4.6Kb. These size relations between the genomes of the λ *trp*51 series, though only approximate, will be useful in comparisons between digests obtained with other sequence specific endonucleases.

(ii) *Analysis with Hind II & III*: Plate 7 shows the digestion products of λ^+ and of λ*trp*51 series' phage DNAs obtained with an unfractionated mixture of *Hind*II and *Hind*III activities, and a λ:*Hind*III digest run for calibration purposes (track 1), as resolved by electrophoresis through a 1% agarose gel. The conditions used for digestion and electrophoresis are fully described in chapter 2.

Plate 7 HindII & III digests of λtrp51 phages analysed on a 1% agarose gel

The λ^+ : HindIII digest is included for calibration purposes, and the λ^+ : HindII & III digest allows the assignment of λ bands in the transducing phage digests. Solid pointers indicate the trp containing fragments in the digest of λtrp^+51 DNA, and the open pointers show the positions of the 'fusion fragments' generated by the deletions.

Track
1. λ^+ : HindIII
2. λ^+ : HindII & III
3. 407.5
4. λtrp^+51
5. λtrp51∇LD102
6. λtrp51∇OE1
7. λtrp51∇BE9
8. λtrp51
9. -
10. λ^+ : HindII & III

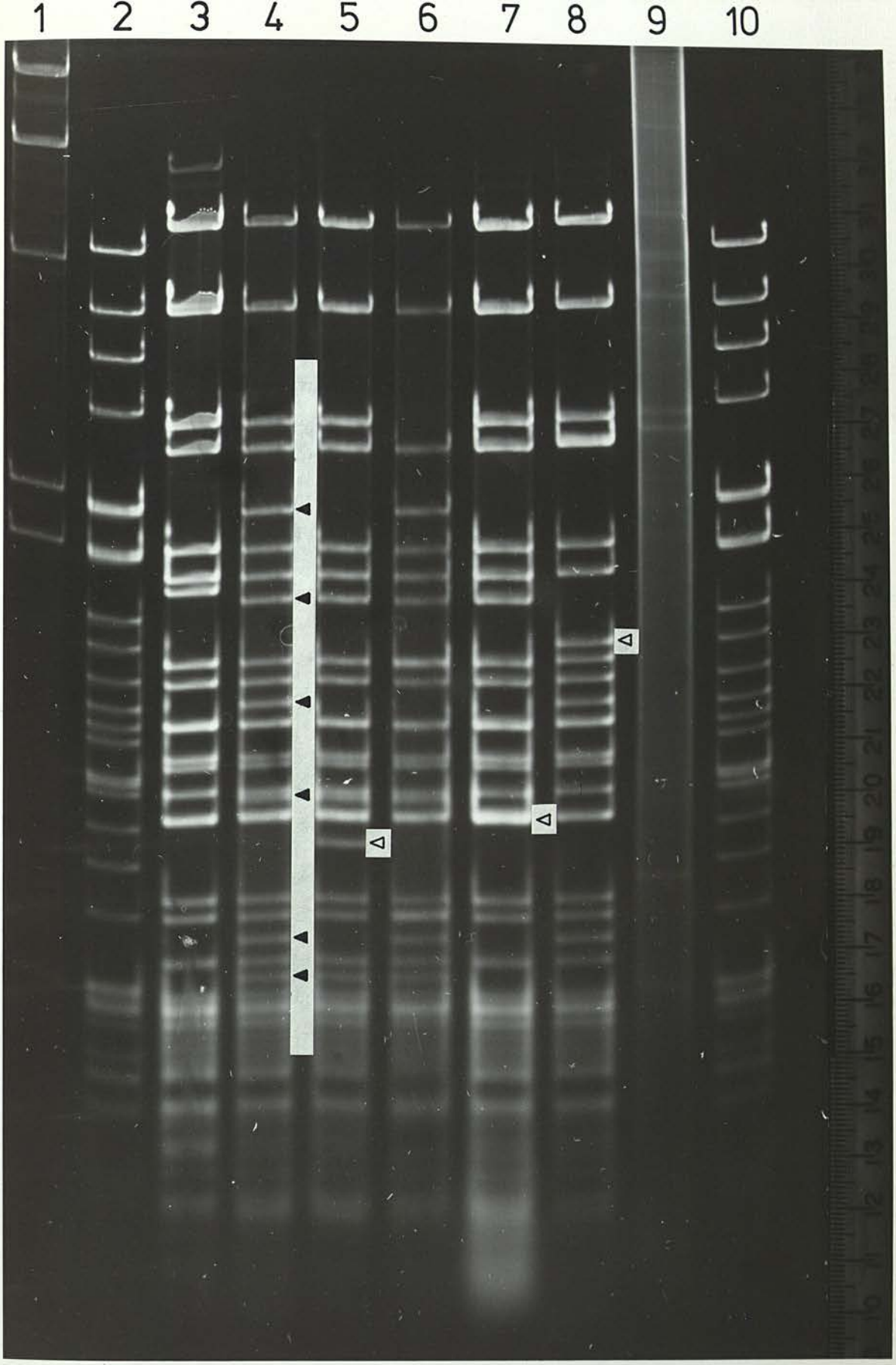


Plate 7

The digests of λ trp$^+$51 and its hybrid parent phage, 407.5, may be compared in a search for those bands which are specific to the transducing phage and thus represent fragments containing bacterial DNA, as explained in Appendix I. The six bands indicated in Plate 7 by solid pointers were identified in this way. The open pointers indicate the new fusion fragments specific to each of the transducing phage derivatives, arising by the juxtaposition of those sections of the two fragments only partially removed by the deletion or substitution. The sizes of these fragments were assessed as described in Appendix II and the values shown in Plate 7 ascribed to each of the six bands with the following estimated errors (again based on an uncertainty of 2mm in measurement of the migration distance).

HindII & III:trp operon fragment	Estimated size	
	Md	base pairs
1	1.55 ± 0.1	2,325 ± 150
2	1.15 ± 0.1	1,725 ± 150
3	0.90 ± 0.05	1,350 ± 75
4	0.70 ± 0.05	1,050 ± 75
5	0.53 ± 0.04	795 ± 60
6	0.45 ± 0.03	675 ± 45

Plate 8 shows the same λ trp51 series:HindII & III digests analysed by a 2.5 - 7.5% linear gradient polyacrylamide gel in order to resolve lower molecular weight fragments and hence detect

Plate 8 HindII & III digests of λtrp51 phages analysed on a gradient polyacrilamide gel

Digests, prepared as before, were submitted to electrophoresis through a linear gradient polyacrilamide gel. The λ^+ : HpaII digest provides additional low molecular weight reference markers.

Track 1. λ^+ : HindII & III
2. 407.5
3. λtrp$^+$51
4. λtrp51 ∇ LD102
5. λtrp51 ∇ OE1
6. λtrp51 ∇ BE9
7. λtrp51
8. λ^+ : HindII & III
9. λ^+ : HpaII

The very small fragment of an indeterminate low MW (less than 250 base pairs) marked '?' is not present in the parental 407.5 digest nor in that of λtrp51 ∇ OE1, but is present in λtrp51 ∇ LD102. It is thus taken as originating from upstream of the trp Leader sequence - the site thus defined is marked with a dotted arrow in Figure 3-3.

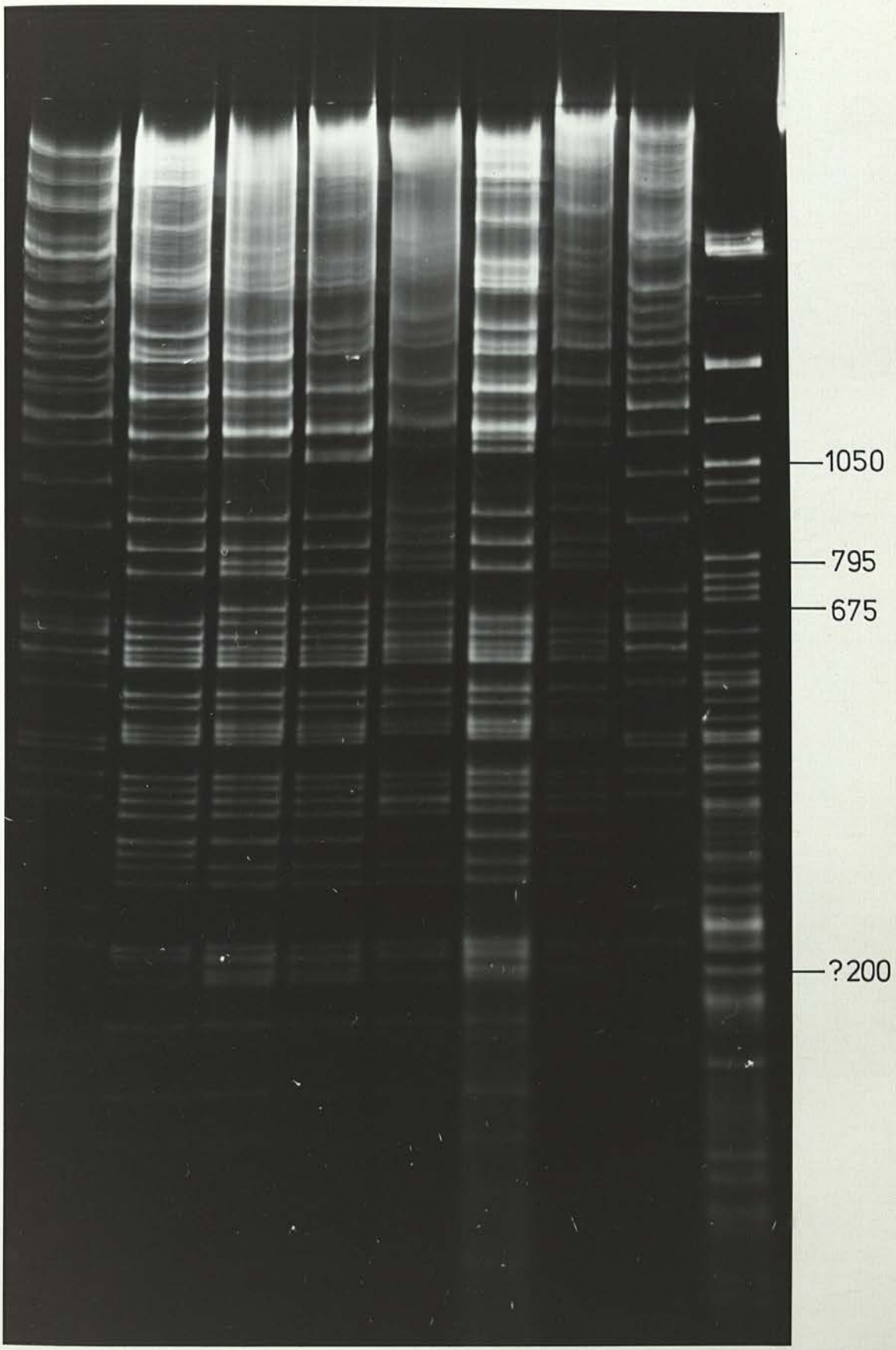


Plate 8

any smaller HindII & III digestion products of trp operon DNA. The gel clearly shows the two smallest trp:HindII & III fragments and the ∇LD102:HindII & III fusion fragment (indicated by pointers in Plate 8), and these, along with the λ:HindII & III fragments of known molecular weight were used to plot a calibration curve of molecular weight vs fragment mobility (Appendix II). The gradient gel has detected and resolved fragments from 1000 base pairs down to a lower limit of approximately 150 base pairs.

The λtrp^{+}51 digest (track 3) shows no new bands in this size range when compared with that of its hybrid parent phage, 407.5, (track 2). It would seem, then, that the above six fragments are the only major products of HindII & III digestion of the trp operon DNA, though fragments of less than 150 base pairs would not have been detected, and fragments producing bands comigrating with any of those already present may have been overlooked.

Comparing the known distribution and overlaps of the standard deletions : Figure 1-1 , carried by the λtrp51 series of transducing phages with the observed presence or absence in the corresponding digest of the six identified HindII & III fragments containing bacterial DNA, a fragment order for the trp operon DNA may be pieced together.

The following table is an 'attendance register' denoting the presence or absence of each of the six trp operon DNA:HindII & III products in digests of each of the λtrp51 series of phage DNAs.

trp deletion carried by λtrp+51 derivative	trp operon:HindII & III fragment present					
	1	2	3	4	5	6
∇AC9	-	-	+	-	+	-
∇BE9	-	+	-	-	-	-
$\nabla\lambda^{LD}$102	-	+	-	+	-	+
∇OE1	+	+	-	+	+	+

The AC9 derivative retains fragments 3 and 5 which must therefore originate from the trpDE segment of the operon, and must necessarily be contiguous. Also fragments 1, 2, 4 and 6 must form another set of unknown order, of which fragment 2 is the only one present in the BE9 derivative and must therefore originate from the trpA end of the operon. This gives an order 2-(1,4,6)-(3,5) in the transducing phage trpABCDE orientation, where parentheses enclose unordered fragments. The LD102 derivative retains fragments 2, 4 and 6, giving the order 2-(4,6)-1-(3,5). Finally, since OE1 removes only fragment 3 from the trpE end of the operon the final order deduced is 2-(4,6)-1-5-3. The fragments 4 and 6 cannot be placed in sequence by the above data, leading to the preliminary trp:HindII & III map shown in figure 3-3.

Figure 3-3 Alignment of genetic and physical maps of the E.coli trp operon

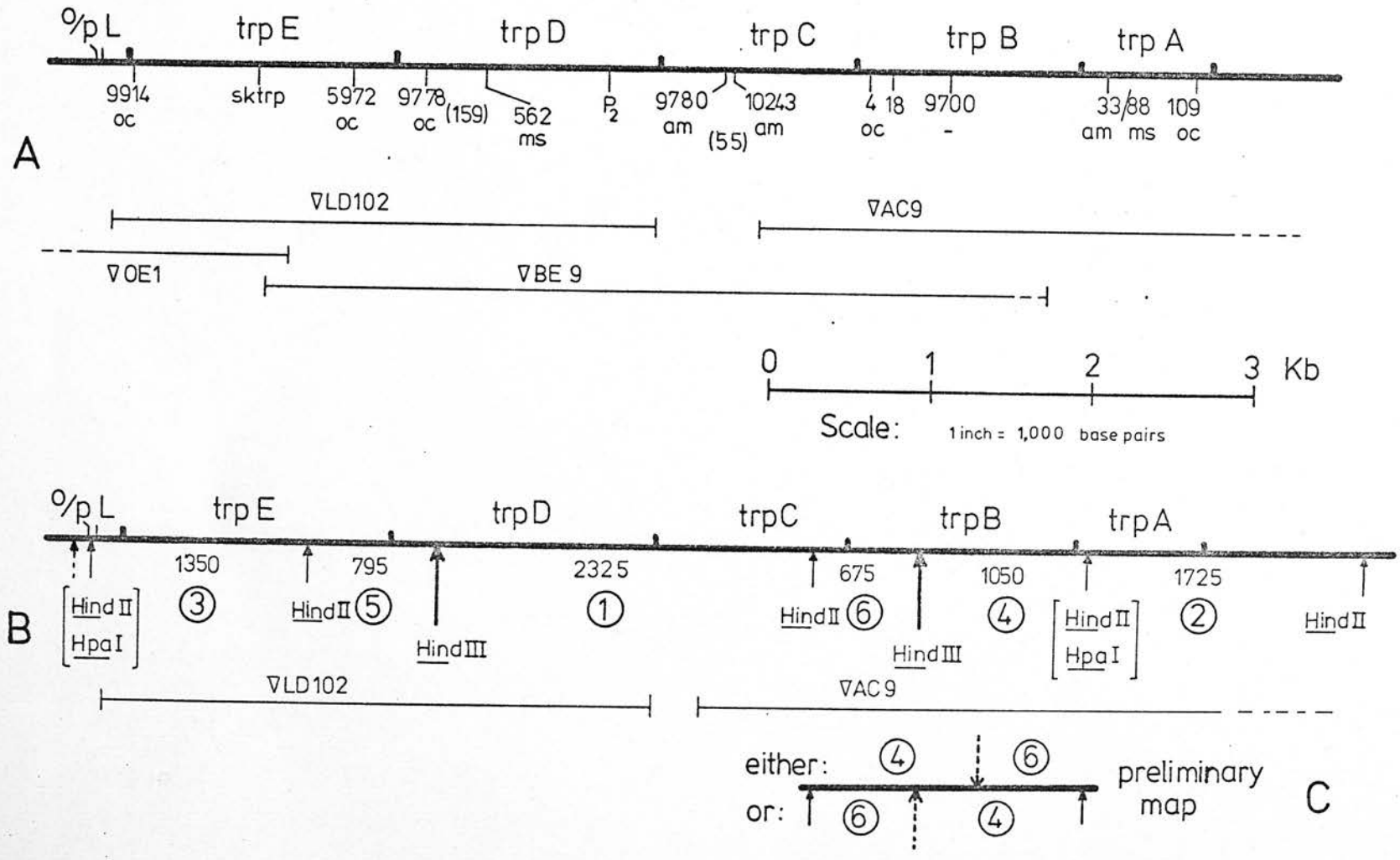
The five structural genes, trpE - A, are presumed to be contiguous, without spacer regions, and to occupy 30 base pairs per 1,100 MW of the determinant proteins (i.e. each 3 base pair codon specifying an amino acid residue of average MW 110 daltons). L is the untranslated leader region at the 5' end of the trp transcript, o/p is the major control complex and P_2 is the minor internal constitutive promoter.

Map A shows the arrangement of point mutations and sites within each gene as determined by genetic linkage data (Yanofsky et al., 1971; Crawford et al., 1970 and Drapeau et al., 1968). The extent of deletions are taken from Jackson & Yanofsky, 1972 (for LD102); Murray & Brammar, 1973 (for OE1 and BE9); and Yanofsky et al., 1971 (for AC9).

Map B shows the refined physical map obtained by superimposing the determined order of restriction fragments on the trp gene lengths (estimated as in A, above). HindII and HpaI targets are marked by fine arrows and the two HindIII targets by bold arrows. The circled numbers denote the trp DNA fragments produced by HindII & III digestion in descending order of size, and the numbers above these circles represent the measured fragment sizes in base pairs.

The preliminary map C, shows the two possible alternative arrangements of fragments 4 and 6 which have been distinguished to produce the refined map B.

FIGURE 3-3 Alignment of genetic & physical maps



(e) Analysis of trp deletions selected with sodium pyrophosphate

In order to complete the HindII & III fragment map of the trp DNA of λtrp$^+$51, more of its deletion derivatives were needed to chance upon one which separates trp:HindII & III fragments 4 and 6. Forty-eight deletions entering the trp operon were selected after pyrophosphate-treatment of λtrp$^+$51, screening the survivors for those (approximately 10% of the total) which displayed a Trp$^-$ phenotype, as described in chapter 2. Of the forty-eight pyrophosphate-selected trp deletions, the following Trp complementation patterns (determined against W3110 carrying trp mutations A88, B9700, C9780C10243, D9778 or E9914) were found:

Trp phenotype	number observed	designation (λtrp51 PP▽ number)
$A^-B^-C^-D^+E^+$	40	1, 4, 5 and 6 .....
$A^+B^+C^+D^+E^-$	3	47, 49 and 50
$A^+B^+C^+D^-E^-$	2	2 and 19
$A^-B^-C^-D^-E^+$	2	27 and 29
$A^-B^-C^+D^+E^+$	1	43

The preponderance of (ABC) deletions may be due to the selection of sibling phage progeny from a single mutant clone, since all the deletions were selected from the same high-titre stock of λtrp$^+$51, or it may reflect an intrinsically higher probability of deletion - formation originating from the trpC gene and the tonB region. This was not investigated further.

The eight λ*trp*51 PP deletions: 1,2,4,5,6,19,27 and 29 were for further study. Phage DNA was purified from each and digested with *Eco*RI (Plate 9) and *Hind*II & III (Plate 10).

The mobilities of the unique *trp* fragments in the *Eco*RI digests were measured and their molecular weights assessed from the calibration curve shown in Appendix II, and the extent of each deletion deduced:

λ*trp*$^+$51PP∇ number	MW of *trp*:EcoRI fragment		estimated extent of deletion in base pairs
	Md	Kb	
λ*trp*$^+$51	11.3 ± 1.0	17.0 ± 1.5	-
PP∇1	7.5 ± 0.7	11.3 ± 1.1	5,700
PP∇2	9.0 ± 0.8	13.5 ± 1.2	3,500
PP∇4	7.5 ± 0.7	11.3 ± 1.1	5,700
PP∇5	7.5 ± 0.7	11.3 ± 1.1	5,700
PP∇6	7.5 ± 0.7	11.3 ± 1.1	5,700
PP∇19	7.8 ± 0.7	11.7 ± 1.1	5,300
PP∇27	9.2 ± 0.8	13.8 ± 1.2	3,200
PP∇29	9.2 ± 0.8	13.8 ± 1.2	3,200

Deletions 1,4,5 and 6 do indeed appear to be identical in extent, though 2 and 19 are different as are 27 and 29. The *Hind*II & III digests of the λ*trp*51PP∇ phage DNAs show complicated restriction fragment patterns, but only the presence or absence of the six *trp*:*Hind*II & III fragments (indicated in Plate 10) need

Plate 9 EcoRI digests of λtrp51PP∇ phages

The sizes of the remaining trp operon containing fragments of the derivative phages may be compared with that of their original λtrp^{+}51 progenitor. All digests were prepared with EcoRI.

Track
1. λtrp51PP∇29
2. λtrp51PP∇6
3. λtrp51PP∇5
4. λtrp51PP∇4
5. λtrp51PP∇1
6. λtrp^{+}51
7. λtrp51PP∇27
8. λtrp51PP∇2
9. λtrp^{+}51
10. λtrp51
11. λtrp51PP∇19
12. 407.5

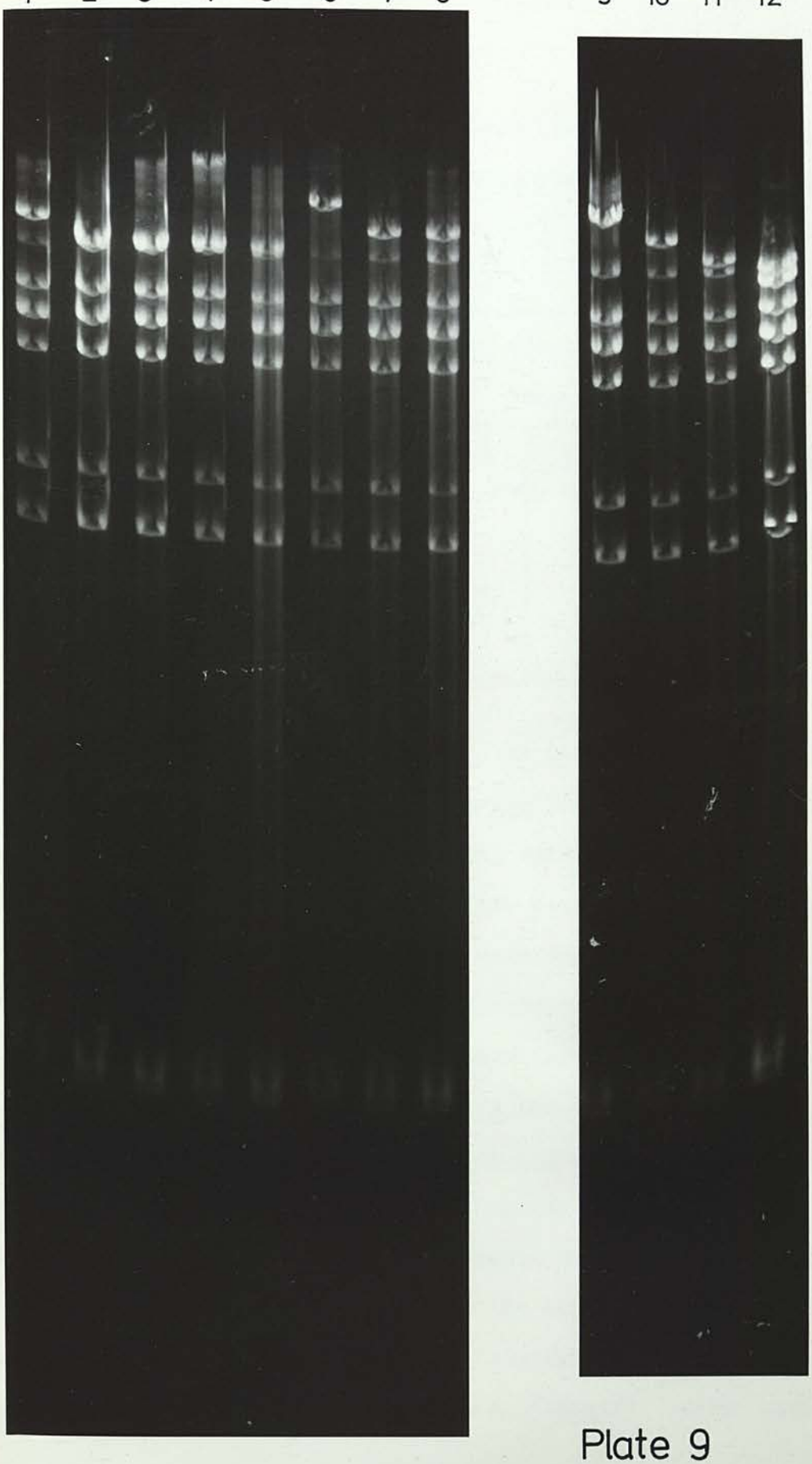


Plate 9

concern us here, these being an effective fingerprint of the trp operon DNA retained by the deletion derivatives. The distribution observed in Plate 10 is as follows:

PP∇ carried by	trp operon HindII & III fragment present					
λtrp⁺51 derivative	1	2	3	4	5	6
PP ∇1,4,5 and 6	-	-	+	-	+	-
PP ∇2 and 19	-	+	-	+	-	+
PP ∇27 and 29	-	+	+	-	+	-

As can be seen from the table, the pairs of deletions ∇2 and 19, and ∇27 and 29 affect the same trp DNA:HindII & III bands, even though they are all demonstrably different. All three classes of deletion remove fragments which are contiguous in the previously determined order (Figure 3-3), but unfortunately, none resolve the sequence of the unordered pair of fragments, 4 and 6. An alternative method was sought to solve this problem.

Definitive information regarding the distribution of cleavage sites for isolated HindIII within the trp operon DNA became available about this time (Hopkins, Murray & Brammar, 1976). It was shown that only two HindIII targets lie within the trp operon DNA sequence, and that these define the termini of a fragment of molecular weight 2.0×10^6 daltons, containing the trpC gene. This trpC:HindIII fragment was cloned in a λ:HindIII vector having a

Plate 10 HindII & III digests of λtrp51PPV phages

HindII & III digests of the PPV derivative phages are compared with that of their λ*trp*$^{+}$51 progenitor. The affected *trp* operon fragments are indicated by the solid pointers.

Track 1. λ*trp*$^{+}$51

2. λ*trp*51PPV1

3. " PPV2

4. " PPV4

5. " PPV5

6. " PPV6

7. " PPV19

8. " PPV27

9. " PPV29

10. λ*trp*$^{+}$51

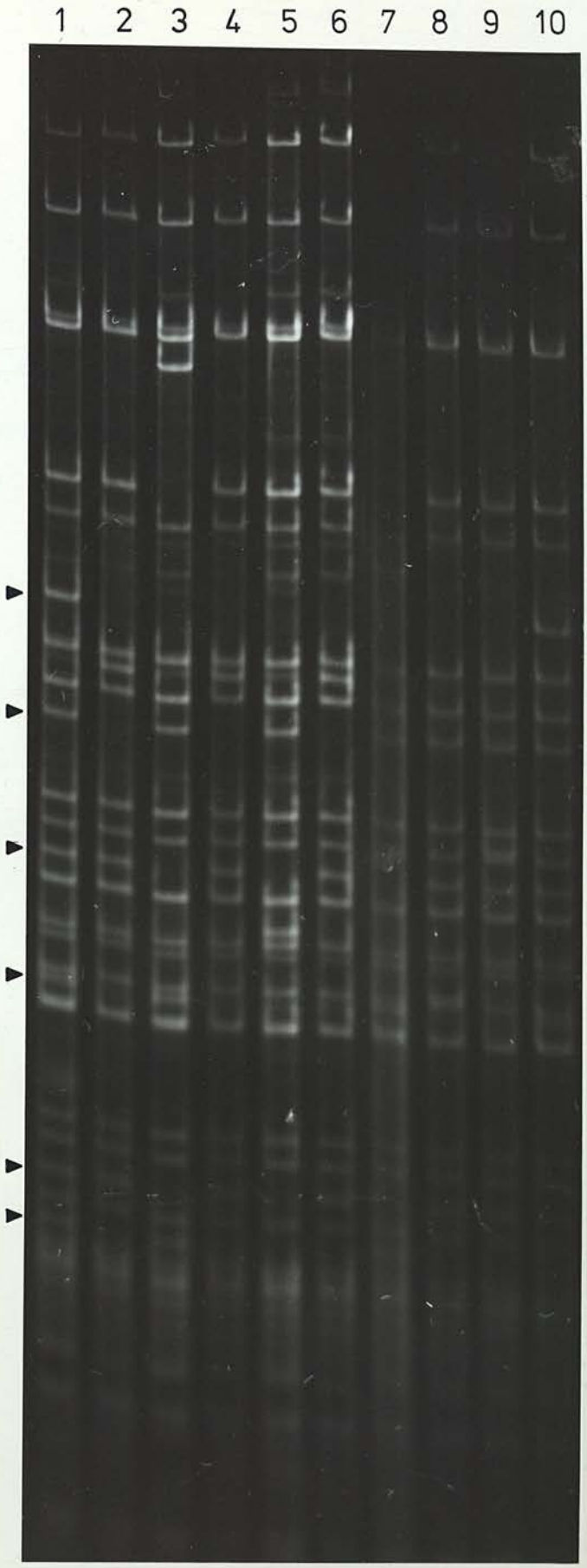


Plate 10

single target for that enzyme. An *in vitro* λ*trpC*$^{+}$ recombinant of this type, λvector540*trpC*$^{\ell}$2, was obtained and its DNA digested with HindIII. Plate 11 shows this digest, in parallel with those of λ^{+} and λvector540 for comparison and calibration. A molecular weight of 2.0 Md was assigned to the trpC:HindIII fragment on the basis of its observed mobility (Appendix II) in reasonable agreement with the published value.

Now, a DNA fragment of molecular weight 2.0 Md contains 3,000 base pairs, and since the same HindIII sites are cleaved by the HindII & III mixture, some fragment or combination of fragments present in the digest of trpDNA by the unfractionated mixture must together form the 3,000 base pair HindIII:*trpC*$^{+}$ fragment. Figure 3-3 shows that bands 6 & 1 (675 + 2325 = 3,000 base pairs) have the requisite total length and these were shown to be produced by HindII digestion of the HindIII:*trpC*$^{+}$ fragment. Thus, since the trp:HindII & III fragments 1 and 6 are present within an isolated HindIII fragment, they must be adjacent to each other. This leads to the final trpDNA:HindII & III fragment order: - 2,4,6, 1,5,3 - shown in Figure 3-3.

(f) Alignment of the genetic and physical maps of trp DNA and refinement of the trp:restriction map

The HindIII mapping already referred to identifies the sites between the trp:HindII & III fragments 4 and 6, and between fragments 1 and 5 as targets for the HindIII enzyme. An alignment of these targets with the trp genetic map has already been achieved (Hopkins, Murray & Brammar, 1976), placing the former (shnIII:trpB) between the loci of mutants trpB4 and trpB18, and the latter (shnIII:trpD) between trpD159 and trpD562.

Bertrand et al. (1975) have obtained the sequence of the first 200 bases of the 5' end of the in vivo trp operon mRNA, overlapping the known trpE N-terminal amino-acid sequence. This sequence, which contains no HindII or HindIII sites in its complementary DNA, was extended by Bennett et al. (1976) to cover the preceding 33 bases. The sequence obtained shows an HpaI site $\begin{matrix}(5'GTT\overset{\downarrow}{A}AC\ 3')\\(3'CAA\underset{\uparrow}{T}TG\ 5')\end{matrix}$ at a position 9-14 bases prior to the start of transcription in the trp operon DNA, and Bennett et al. went on to show that this site was indeed cleaved by the HpaI endonuclease and could be protected from such attack by pre-bound trp repressor or RNA-polymerase, thus locating it within the operator/promoter control region.

Since the HpaI recognition sequence is a subset of that of HindII $\begin{matrix}(5'\ GTY\overset{\downarrow}{R}AC\ 3')\\(3'\ CAR\underset{\uparrow}{Y}TG\ 5')\end{matrix}$ the HpaI site in the trp operon should have been cleaved by the HindII & III mix. Also, since there are no HindII or HindIII recognition sequences within the next 200 bases on the trpE side, no small fragment outside the limits of detection

of the present work should be present. Thus, the only candidate for the sHpaI amongst those HindII sites mapped in the previous section is that defining the end of trp:HindII & III fragment 3, distal to fragment 5.

The single trp:HpaI site and the two trp:HindIII sites together allow only one possible alignment of the physical and genetic maps of the trp operon as shown in Figure 3-3.

To test for the presence of other HpaI targets within the trp operon, DNA samples of the λtrp51 series of phages were digested with this enzyme. Plate 12 shows the fragment patterns obtained. The measured λ:HindIII fragment mobilities were used to draw a calibration curve (Appendix II) from which the molecular weights of the unique HpaI bands of each of the λtrp51 digests, indicated by pointers in Plate 12, were determined as follows:-

Plate 12 Phage DNA	Size of trp:HpaI fragments Kb (kilobase pairs)	Net structural change, Kb	Sum Kb
Track 3 λtrp$^+$51	3.22 + ?	-	?
Track 4 λtrp51∇102	3.22 + 2.48	∇102 : 3.6	9.3
Track 5 λtrp51 (i.e. ∇AC9)	- 5.92	∇AC9 : 3.4	9.3
Track 6 λtrp51∇BE9	3.22 + 1.02	∇BE9 : 5.3	9.5

Plate 12 HpaI digests of λtrp51 phages

The λ^+ : HindIII digests are included for reference. All other digests are with HpaI. Solid pointers indicate trp operon fragments in the digest of the trp^+ phage. The open pointers show the positions of the 'fusion fragments' generated by the trp deletions LD102, AC9 and BE9.

Track
1. λ^+ : HindIII
2. 407.5
3. λtrp^+51
4. λtrp51∇LD102
5. λtrp51
6. λtrp51∇BE9
7. λ^+ : HindIII

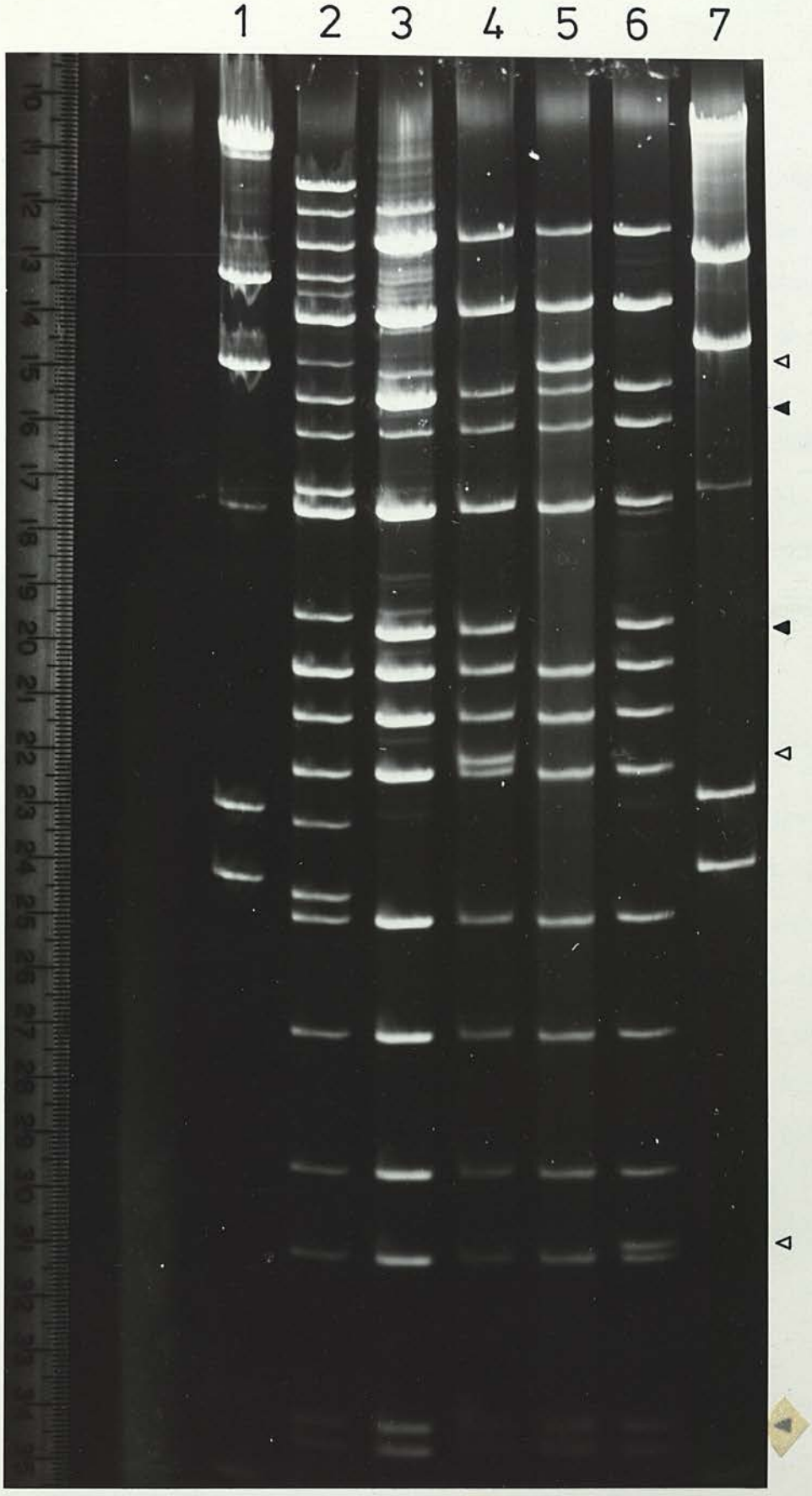
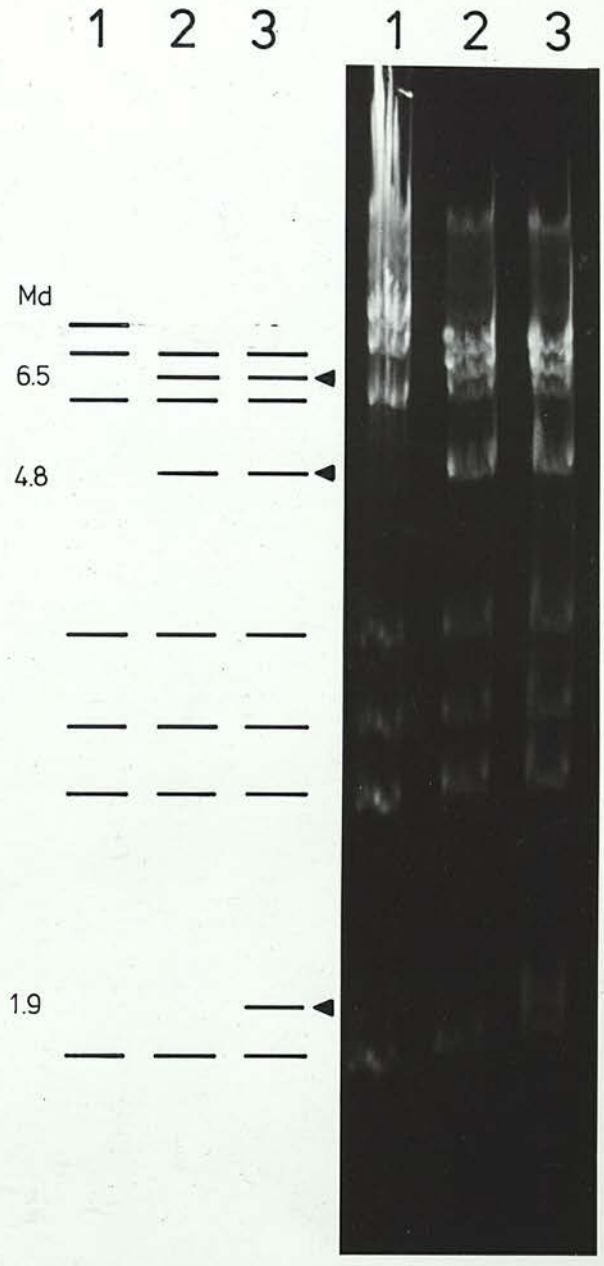


Plate 12

Since the sum of the trp:HpaI fragments together with the net size change of the deletions or substitutions with respect to λ trp$^+$51 should clearly be equal, it can be deduced from the above table that the λ trp$^+$51 digest should also contain a fragment of size (9.3 - 3.22), 6.1Kb in length. The calibration curve plotted in Appendix II shows that such a fragment would be found at position approximately 15.5cm on the gel shown in Plate 12. A band at position 15.7cm is present in all the digests, and thus probably overlaps and obscures a trp$^+$:HpaI band in the λ trp$^+$51:HpaI digest (Plate 12, track 3).

The deletion/substitution pattern shows that the 3.22Kb fragment is removed only by ∇AC9 (see the above table), and this originates from the trpA end of the operon. The 6.1Kb fragment, which ~~is augmented by~~ contains ∇BE9 and ∇LD102, must therefore span the distance between the known HpaI site in the trp operator/promoter region and one of the HindII sites in the trpA region of the operon. By measuring 6.1Kb into the trp operon DNA from the position of the known HpaI site on the physical map (Figure 3-3), the estimated position of the second HpaI site in the operon falls within 100 base pairs of the mapped position of the HindII site between trp:HindII & III fragments 2 and 4. Since the HpaI recognition sequence is a subset of that of HindII, the latter two sites are presumed to coincide.

Plate 13 shows the banding patterns of the hybrid, 407.5 and its progeny phages λ trp51 and λ trp$^+$51 digested with HindIII. Since the gel is of poor quality, the schematic diagram in parallel,



Hin d III digests:

1. 407.5
2. λ trp 51
3. λ trp^{+} 51

Plate 13

shows the banding changes. A single band is lost from the 407.5 digest and substituted by two smaller bands in that of λ*trp*51. The λ*trp*$^+$51 digest differs discernably from that of λ*trp*51 only by the acquisition of one new band. These changes are indicated in Plate 13, and their approximate molecular weights shown in the schematic representation alongside.

The unique HindIII band in the *trp*$^+$ digest (track 3) is consistent with a fragment size of 1.9Md, the published value for the *trpC*$^+$:HindIII fragment. No bands corresponding to the other two Ecotrp:HindIII fragments (of molecular weights 3.8Md and 2.0Md, carrying *trpE*$^+$ and *trpA*$^+$, respectively: Hopkins, Murray & Brammar, 1976) were observed however, and the conclusion was drawn that the HindIII sites in the bacterial DNA immediately adjacent to the *trp* operon are not present in λ*trp*$^+$51. That the site preceding *trpE* and the *trp* operator & promoter region is absent is not surprising, on the basis of the DNA content of λ*trp*$^+$51 (Figure 3-2), but the estimated position of the HindIII site downstream of *trpA* is only 400 base pairs downstream of the last mapped HindII & III target (Figure 3-3). Since this is absent, two conclusions can be drawn: first, the mapped HindII & III site at the *trpB* distal end of *trp*:HindII & III fragment 2 (Figure 3-3), is cleaved by HindII, and secondly the bacterial DNA carried by λ*trp*$^+$51 falls short of the known HindIII sequence downstream of *trpA*.

The sequence-specific endonuclease AvaI, from the blue-green alga Anabaena variabilis recognises and cleaves the sequence
5'-C↓YCGRG-3'
3'-GRGCYC↑-5'
in double-stranded DNA and its sites within the Lambda genome have been mapped (Hughes, 1977 : Figure 1-2). In order to test for the presence of an AvaI site (or sites) in the trp operon DNA the by now familiar analysis of digests of the λtrp51 series DNAs was performed : Plate 14.

The λtrp$^+$51 digest (track 5) has a unique large fragment with an estimated molecular weight of 5.2 ± 0.3Md, corresponding to 7.8 ± 0.45Kb. This fragment is missing from all of the other digests of the λtrp51 series, and must therefore carry most of the trp operon DNA; since, however, no other fragment is detectably affected by the incorporation of the deletions BE9 and LD102, (which together span most of the operon) and the only other fragment affected by the AC9 deletion is very small (ca. 450 base pairs), I conclude that most, if not all of the trp operon lies within the 7.8Kb AvaI fragment with the possible exception of a small region of trpA. The actual positions of the two AvaI sites flanking the operon in λtrp$^+$51 may be deduced by taking the known locations of the λ:AvaI targets as fiducial points.

The order of the AvaI fragments in the intact lambda DNA molecule is D'-A-H-C-E-F-D-G-B, labelled alphabetically according to size from the largest product : A, to the smallest : H, where D and D' are indistinguishable in size. Only fragments D and G of the wild-type lambda digest are retained in that of λtrp$^+$51 (Plate 14), fragments D'-F must have been lost by the substitution of Ø80

Plate 14 AvaI digests of the λtrp51 series of phages

EcoRI and AvaI digests of wild-type lambda DNA are included for calibration and reference.

The single trp operon containing fragment in the digest of λtrp$^+$51 is at position 11.0 cm on the scale (track 5).

Track	1 & 2	λ$^+$: AvaI
	3	407.5
	4	ø80$^+$
	5	λtrp$^+$51
	6	λtrp51▽LD102
	7	λtrp51
	8	λtrp51▽OE1
	9	λtrp51▽BE9
	10	λ$^+$: EcoRI
	11	λ$^+$: AvaI

The smallest trp operon DNA fragment at position 31.5 cm on the gel is best revealed by densitometry - see Appendix II, Figure A-6. This fragment, of an estimated 450 base pairs, is present in AvaI digests of all the λtrp51 series except λtrp51 itself and the hybrid 407.5; it thus originates from the trpA end of the bacterial DNA carried by these phages, as shown in Figure 3-4.

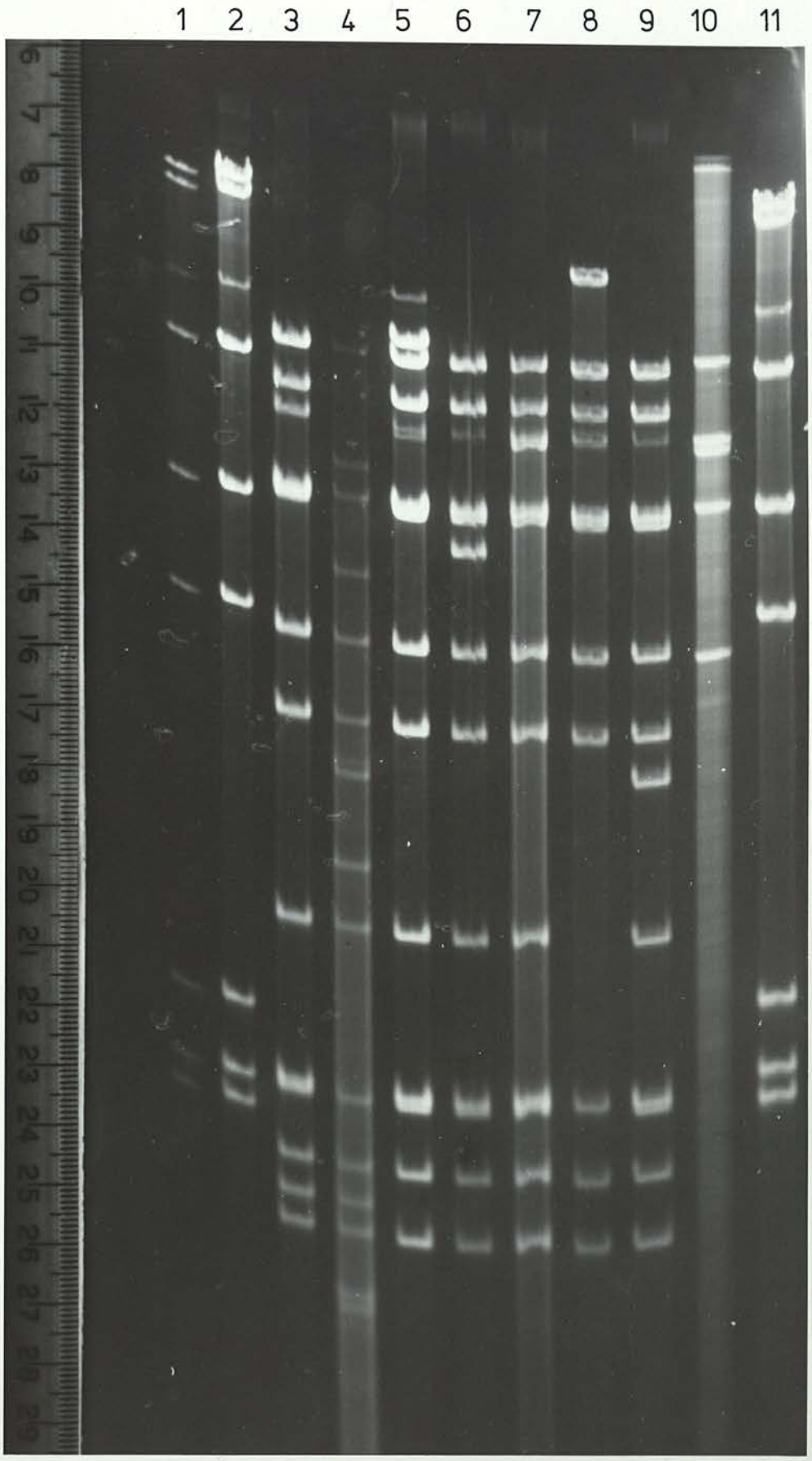
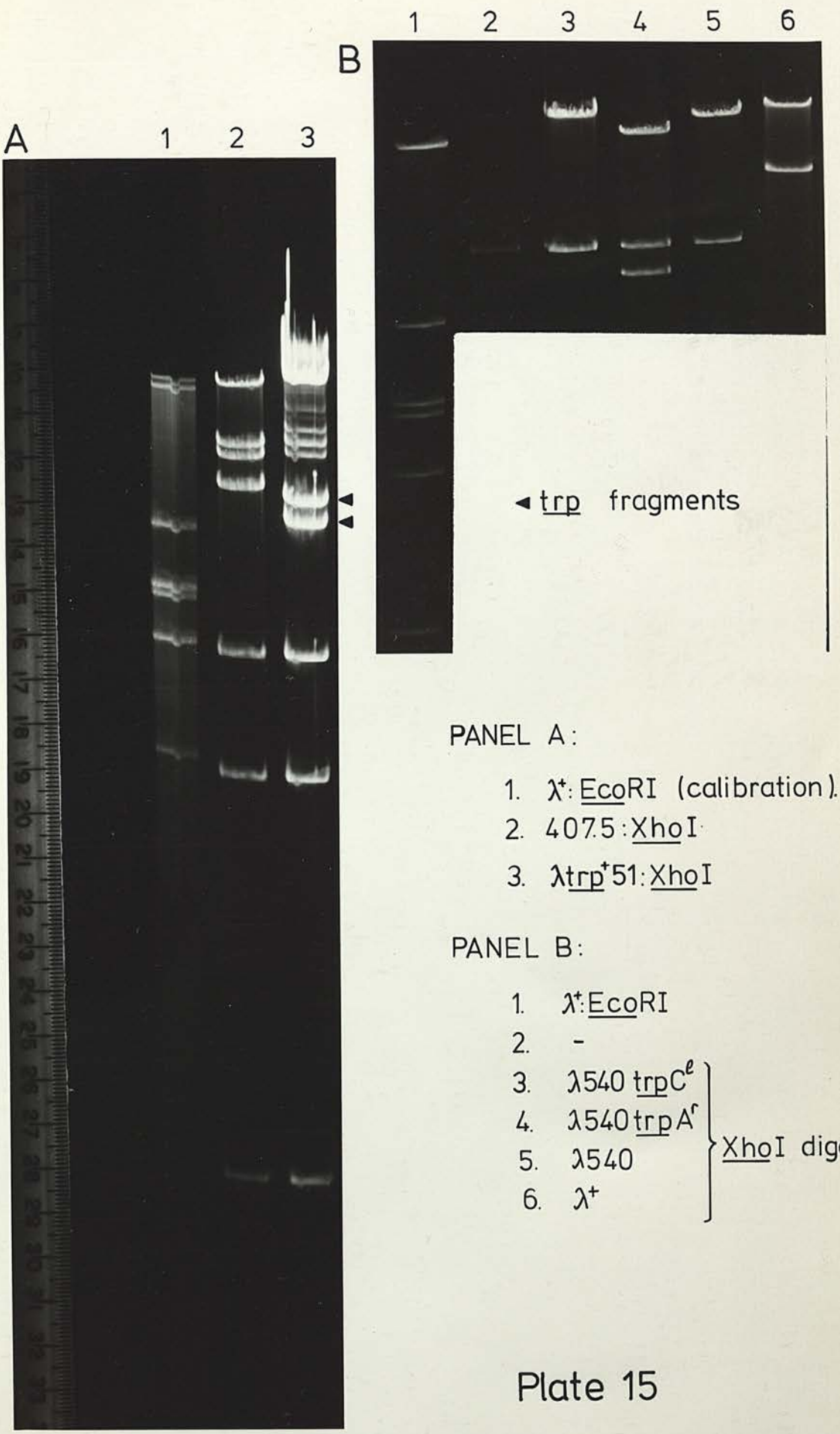


Plate 14



PANEL A:

1. λ⁺: EcoRI (calibration).
2. 407.5 : XhoI
3. λtrp⁺51 : XhoI

PANEL B:

1. λ⁺: EcoRI
2. -
3. λ540 trpC^{e}
4. λ540 trpA^{r}
5. λ540
6. λ⁺

} XhoI digests

Plate 15

material and fragment B is reduced by the ninR5 deletion. Since fragment F is missing, the AvaI target defining its left end-point must lie under the Ø80 DNA in the transducing phage. This places the Ø80 : λ cross-over point in the hybrid to the right of this AvaI site at 65.2% on the standard lambda map, and to the left of the EcoRI site at 65.6% (the latter being retained in the hybrid, as described at the beginning of this chapter). See Figure 1-2.

We can ask whether the Ø80 DNA in the xis-red region of the transducing phages brings with it any new AvaI sites by comparing the λ trp$^+$51 and λ trp51 ∇ OE1 digests, since the OE1 substitution has been shown to completely remove this Ø80 DNA segment. The λ trp51 ∇ OE1 digest (Plate 14 : track 8) shows the loss of the 7.8Kb trp band and also the loss of one other band (due to a fragment of molecular weight 1.25 ± 0.1Md; 2.9 ± 0.15Kb) which must therefore originate from a position between the trp fragment and the lambda fragment D, defining a new site in the Ø80 (xis-red) DNA. The arrangement of AvaI sites shown in Figure 3-4 is thus determined.

The endonuclease XhoI from Xanthomonas holicola, a plant pathogen, cleaves double-stranded DNA at the sequence $\overset{5'\text{-}C\downarrow TCGAG\text{-}3'}{3'\text{-}GAGCT\uparrow C\text{-}5'}$ and thus recognises a subset of the AvaI targets $\overset{5'\text{-}C\downarrow YCGRG\text{-}3'}{3'\text{-}GRGCY\uparrow C\text{-}5'}$.

To test whether ~~(the)~~ either of the AvaI targets in the trpA region of λ trp$^+$51 ~~are~~ is also recognised by XhoI, the XhoI digests of this phage and that of its hybrid parent, 407.5, were compared (Plate 15A). A large fragment in the 407.5 digest is replaced by two smaller fragments in the λ trp$^+$51 digest (indicated by pointers in Plate 15A), and therefore the bacterial DNA carried by the trp$^+$

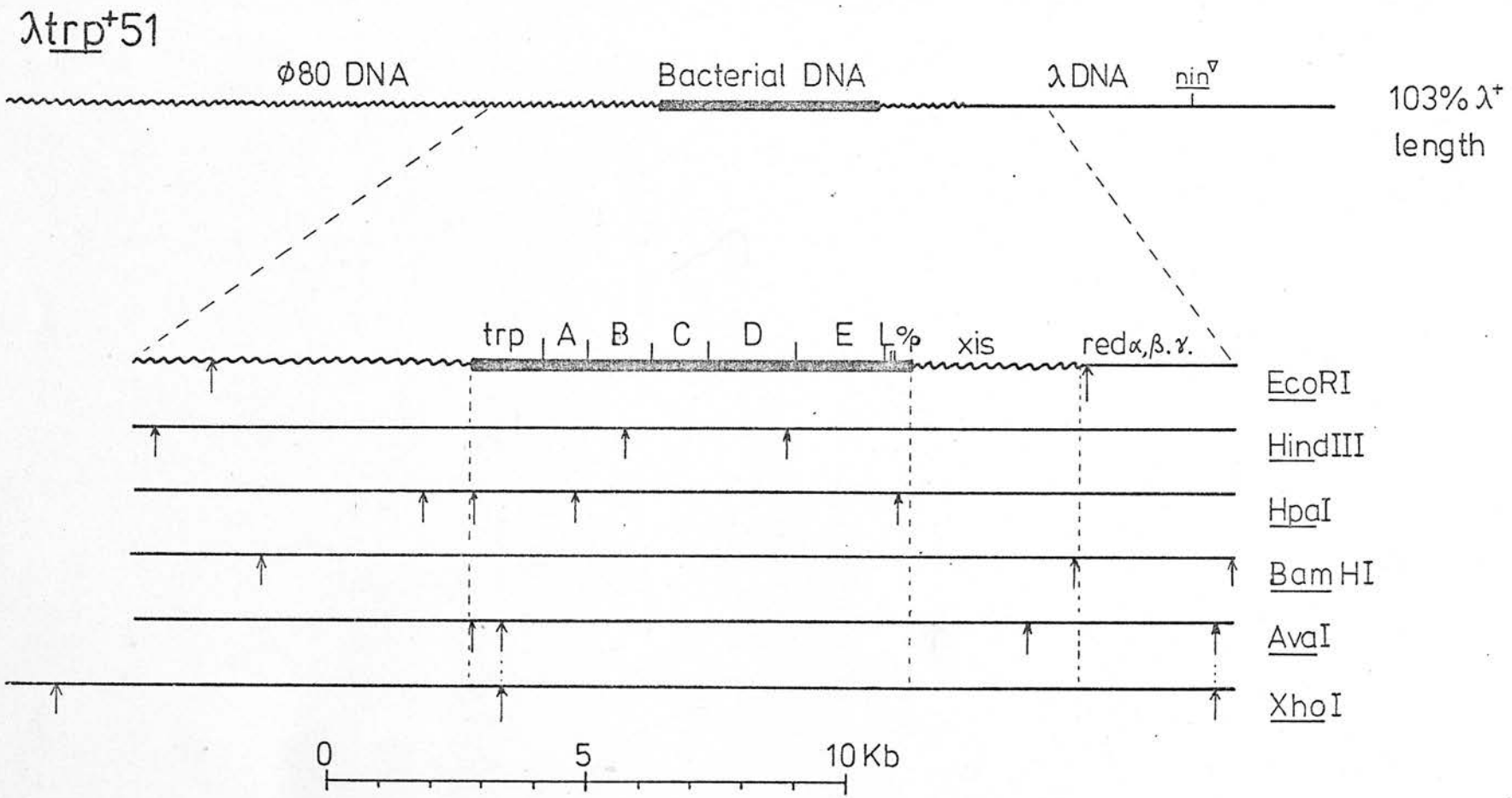


FIGURE 3-4 Restriction targets flanking the trp operon in λtrp+51

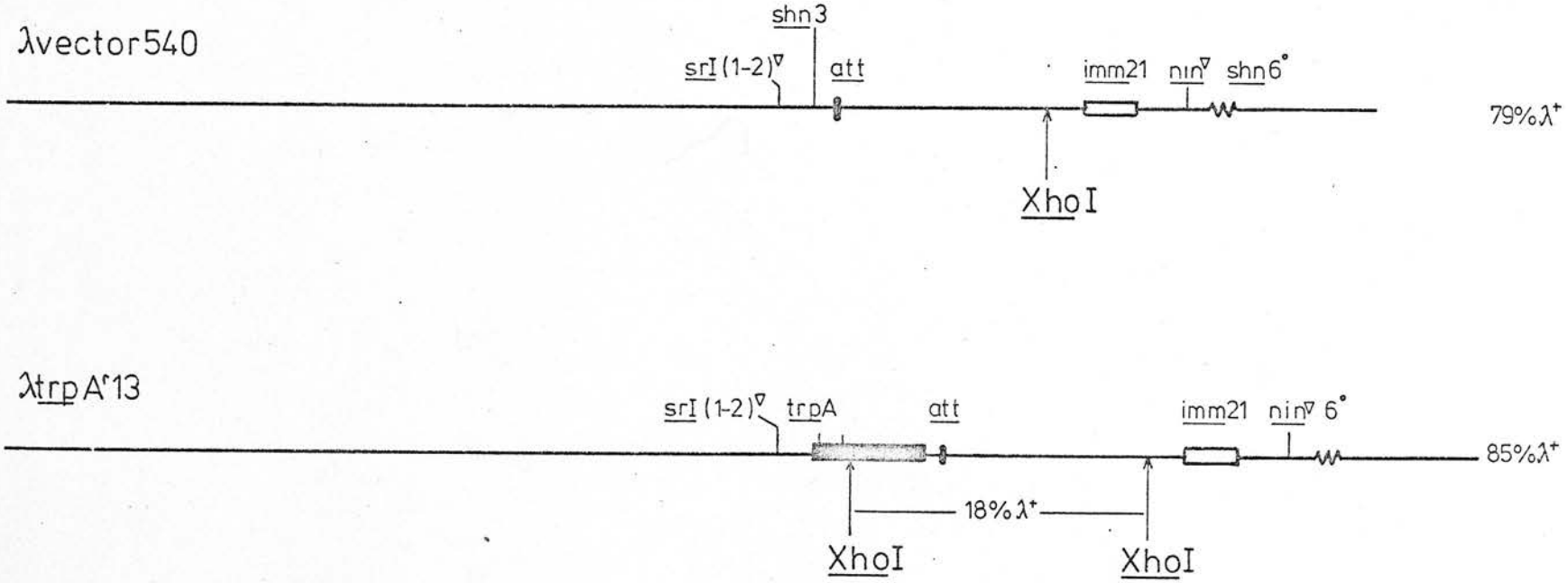


FIGURE 3-5 XhoI targets in λvector540 and λtrpA^r13

phage brings with it an XhoI target. This must be coincident with one of the two observed AvaI targets as shown in Figure 3-4.

To show that the XhoI target is in the trpA proximal region of the bacterial DNA, and not distal to the tonB deletion known to be present in λtrp^{+}51 (see earlier sections of this chapter), the phage λ540trpA^{r}13 carrying the intact TrpA^{+}:HindIII fragment was digested with XhoI, giving the restriction pattern shown in Plate 15B, track 4. That of the vector λ540 alone is shown in track 5, whilst the λ540trpC^{1}2 : XhoI digest in track 3 identifies the common right arm fragment. Since the trpC fragment cannot contain an AvaI target, nor can it harbour an XhoI target.

The two bands seen in track 5 are indicative of a single XhoI site in the DNA of the vector phage : sequencing studies of the 5'-terminal nucleotides of restriction fragments show this to be identical to lambda : AvaI site 6 at 69.4% on the standard map (S.G. Hughes, personal communication) and this is consistent with the lambda : XhoI fragment sizes seen in Plate 15B, track 6.

The λ540trpA^{r}13 digest, however, shows three clearly resolved bands and must therefore arise by the cleavage of two sites recognised by XhoI, one of which must reside within the bacterial DNA (Figure 3-5). The measurements taken from Plate 15B suggest that the bacterial XhoI site is in the trp-proximal segment of the trpA^{+} : HindIII fragment.

The sequence 5'-G↓GATCC-3' / 3'-CCTAG↑G-5' occurs five times in the lambda genome. It is recognised by an endonuclease : BamHI, purified from Bacillus amyloliquefaciens and cleaved at the positions shown. These five BamHI recognition sites have been mapped by Haggerty & Schlief (1976). To detect any BamHI targets within the trp operon DNA, digests of λtrp$^+$51 and its hybrid parent 407.5 were compared (Plate 16), along with the BamHI digests of λtrp$^+$51hλ and of lambda itself.

The hybrid 407.5 to λtrp$^+$51 transition results in the loss of two bands and the acquisition of a single larger band. Thus the small segment of ø80 (att-int) DNA lost in the substitution event contains a BamHI site, and is replaced by a larger (trp$^+$) segment devoid of BamHI sites. See Appendix I for the general argument. Thus the trp operon DNA does not contain a BamHI recognition sequence.

Measurement of the size of the Bam : trp$^+$ fragment together with the known distribution of sites in lambda DNA, reveal the arrangement shown in Figure 3-4.

BamHI digests:

1. 407.5
2. λtrp⁺51
3. λtrp⁺51h^λ
4. λ⁺

Plate 16

CHAPTER 4

Detection of chi-like sites in the E.coli trp operon DNA

The estimation of a length of an internal segment of the *trp* operon on the basis of genetic data on the one hand, and direct physical measurement on the other, have led to widely differing values for the distance between the nearest endpoints of the *trp* deletions LD102 and AC9 (Chapter 3 and compare the data of Yanofsky *et al*, 1971). The respective estimates ~~being~~ are 270 and 600 base pairs : a difference too great to be explained by errors alone.

Such a discrepancy could be explained if the genetic map were distorted in this region by a locally increased frequency of recombination overlapping the measured interval. Thus, a short stretch of DNA with this increased recombination frequency per unit length would, by genetic analysis, resemble a longer stretch of DNA with the normal, lower and uniform level of recombination throughout its length, the physical data being unaffected. A precedent for such sites of locally enhanced frequency of genetic exchange has been observed in the genomes of λ*red*⁻*gam*⁻ phages carrying particular point mutations and in certain λ*pbio* transducing phages (McMilin *et al*. 1974). Stahl defined these sites which initiate an increased local level of recombination as *chi* sites (cross-over hotspot instigators). Several aspects of the effects of *chi* sites are described in the introduction to this thesis.

The properties of *chi* sites, taken together with the above observations were suggestive of a naturally occurring *chi* site in the vicinity of the *trpC* gene, and this chapter describes experiments undertaken to detect and locate any such sites which may be present.

Stahl and co-workers (Stahl *et al.* 1974, and Lam *et al.* 1974) have directly demonstrated an enhancing effect of *chi* sites on the frequency of recombination in the region flanking the *chi* site itself, the stimulation being greatest at the position of the site and falling off with increasing distance from it (though possibly more markedly in one direction than the other), the maximal effect being over ten fold, as judged by density transfer crosses (McMilin *et al.* 1974) and extending over 50% of the lambda genome.

Chi mutations have been shown to greatly enhance the growth of λ *red*⁻ *gam*⁻ phages (Henderson and Weil, 1974) and this is thought to be mediated by a stimulation of host *rec*-mediated dimerisation of monomeric lambda circular DNA (Enquist and Skalka, 1973); the latter is inert in the maturation and packaging system specified by the phage, whereas the former is quite efficiently matured and encapsidated. This assay for *chi* sites was used to characterise the *trp* operon DNA of various specific transducing phages, but since it is only a secondary manifestation of a *chi* site and does not directly prove a role for enhanced recombination, the sites responsible for increasing the vigour of these λ *red*⁻ *gam*⁻ phages will be referred to as *chi*-like, allowing for the possibility that other sites may confer the same phenotype by a different mechanism. This distinction must be maintained until the effect of these *chi*-like sites on recombination has been demonstrated more directly.

The λ *trp* transducing phages constructed in vitro with the restriction enzyme HindIII provide a series of phages which differ only in their content of bacterial DNA. Hopkins *et al.*

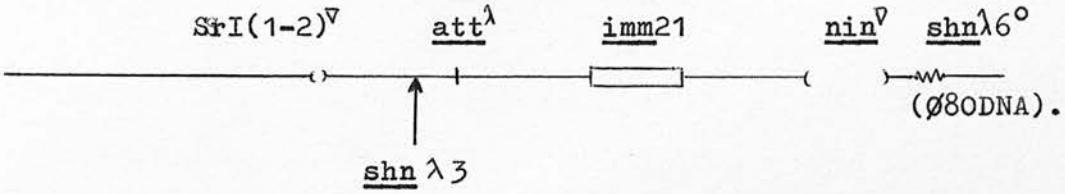
(1976) isolated λ*trp*A, λ*trp*C and λ*trp*E phages by this method, the respective *trp*DNA : *Hin*dIII fragments of length 3.0Kb, 2.85Kb and 5.7Kb being inserted into λ vector 540, at the single remaining *Hin*dIII target (*shn*λ3) in either of the two possible orientations.

Two methods of screening these transducing phages for *chi*-like activities were employed, each based on stimulation of growth of phenotypically Red$^-$Gam$^-$ phages on a *rec*$^+$ host: Any differences revealed between the λ540*trp* series of phages can then be directly ascribed to the transduced bacterial DNA. Both methods rely on the blocking of λ:N gene function and hence of the N-dependent expression of the *red* and *gam* genes: the first by growth of a double amber mutant (*Nam7am53*) on a suppressor-free host, and the second by growth of the phage carrying a wild-type λ:N gene on a host insensitive to λ:N-protein (*groN785*). Both systems require that the phages carry a mutation allowing N-independent expression of the lambda late functions, in this case the deletion *nin*R5.

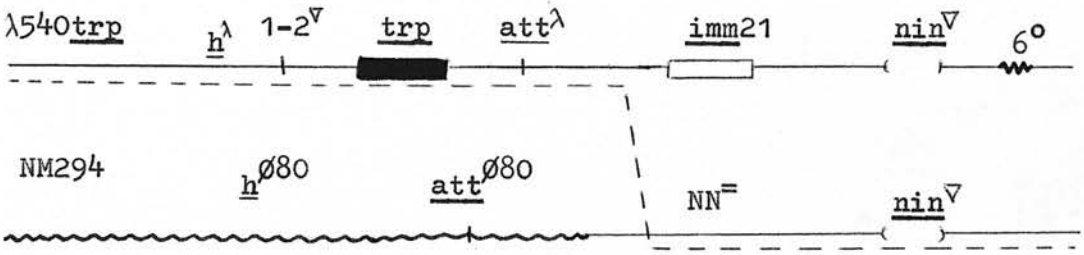
(a) *Growth of λ NNnin phages on a suppressor-free host*

The λ *trp* transducing phages constructed *in vitro* are derivatives of the λ *imm*21-vector NM540 whose constitution is shown below:

λ vector540



The individual transducing phages were constructed by insertion of the bacterial trp operon DNA : HindIII fragments at the position indicated by the arrow, shn λ3. The mutant λ Nam7am53 gene was introduced into each of the transducing phages for the chi test by the following cross:

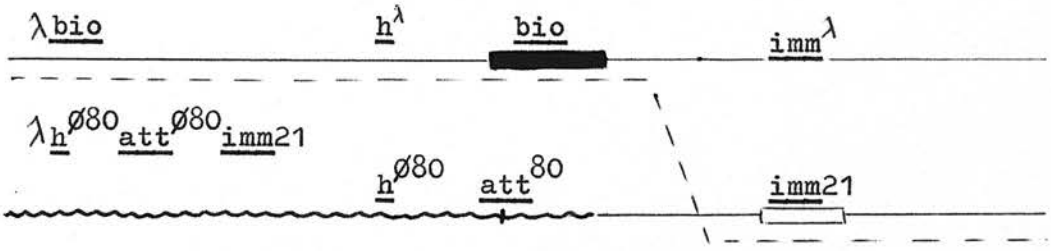


The λ trpNNnin recombinants arising by the indicated exchange were in each case selected as λ h^λ imm^λ phages by growth on the supE strain C600(λ imm21) and purified by single plaque isolation on C600 itself.

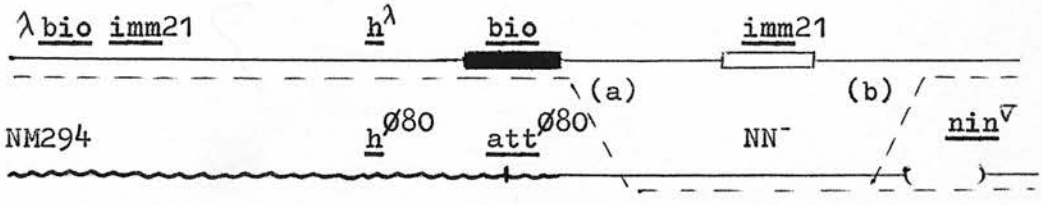
The DNA of the longer λ pbio phages are known to carry a chi site and λ bio16A and λ bio72 were used as control phages:- subsequently both have been shown to carry the bio-chi site (Malone & Chattoraj, 1975), though λ bio16A red⁻ gam⁻ gives a low burst-size, normally indicative of a phage lacking such a site : in this case because of a 3.8% excess of DNA over that of wild-type lambda, since the deletion b519 restores the phage to a larger burst-size.

phage	deletion	addition	net size (% λ⁺)	reference
λ bio16A	1.5%	5.3%	103.8%	Malone & Chattoraj (1975)
λ b519 bio16A	7.8%	5.3%	97.5%	Malone & Chattoraj (1975)
λ bio72	8.6%	8.5%	99.9%	Hradecna & Szybalski (1969)

Thus, λbio16A serves as a pseudo-χ^- control and λbio72 as a χ^+ control. The NNnin genotype was introduced into each of these phages first by constructing the λ bio imm21 and then replacing imm21, containing the phage 21 N gene with imm λ NN in the following two crosses:



λh^λ imm21 recombinants were selected on C600(λ) and purified on C600. These λ bio imm21 derivatives were then crossed to NM294 :



Several λimm^λ recombinants were selected on C600 (λ imm21) as before, and purified on C600. These recombinants were then grown on a suppressor-free host (W3350) to check for the presence of the deletion ninR5, rendering phage growth N-independent. Phages which gave no lysis within a spot containing 10^6 pfu were rejected, having suffered the double recombination event (a).(b), whereas the remainder had undergone the single exchange (a), and one such recombinant from each of the λ bio phages was kept as λ bioNNnin.

The burst-size of each of the λNN*nin* derivatives during growth on the suppressor-free host W3350 was determined as described in 'Methods'. The results are presented in Table III, and schematically in Figure 4-1. The figure shows that λ*c*I857 with its wild type N gene and N-dependent controls, gives a typically high burst size of over 100 pfu per infected centre, whereas λ540NN*nin* (which was predicted to lack a *chi*-site) has a burst size of about 20 : a five-fold reduction as compared with the wild type.

In each case the plaque size of the λNN*nin* derivative on lawns of W3350 parallels the measured burst size, those forming small plaques giving single bursts of about 20 pfu, whereas those forming larger plaques generally give bursts of 60 - 70 pfu per infected cell. The slightly lower burst size of the λ*trp*EDC^{1} phage might be due to its greater DNA content as compared with the other *trp* derivatives, though this cannot be the basis of any difference between λ*trp*EDC^{1} and λ*trp*EDC^{r}. The low value observed for the burst of the λ *bio* transducing phages may reflect the variability of single determinations, though the possibility remains that some quality of the *bio*-*chi*-site differs from that of the *trp* *chi*-like sites leading to significantly different burst sizes under these conditions. The reason for the exceptionally high burst size of the λ*trp*2C^{1}NN*nin* phage, however, is not apparent.

Phage λ*trp*1E^{r}NN*nin* appears to be a special case, as this isolate behaves differently from the other three λ*trp*E^{r} derivatives even though they appear to be identical in *trp*E

Table III : Burst sizes of λNNnin phages on W3350

Phage	%λ length	Experiment I	Experiment II	Experiment III	Mean B.S.	Plaque size on W3350
λcI857 (N^+nin^+)	100	57	138	130	108	large
λ540NNnin	83.5	17	24	22	21	small
λbio16A NNnin	98	24	-	-	24	small
λbio72NNnin	94	41	-	-	41	large
λtrp1A^lNNnin	89.5	19	30	-	25	small
λtrp13A^rNNnin	89.5	19	27	-	23	small
λtrp2C^lNNnin	89.5	104	97	80	94	large
λtrp15C^rNNnin	89.5	36	89	46	57	large
λtrp17E^lNNnin	95.5	-	64	57,73	65	large
λtrp3,5,8E^rNNnin	95.5	-	54	78,81	71	large
λtrp1E^rNNnin	95.5	8	19	30	19	small
λtrp1017EDC^{l}NNnin	101.5	43	44	34	40	large
λtrp1710EDC^{r}NNnin	101.5	56	67	59	61	large
λtrp19ABC^{l}NNnin	96	50	92	-	71	large

Plaque size of the phages produced in the single burst experiment was assayed by spot titration on lawns of W3350 seeded in a soft agar overlay on BBL agar. Two determinations of the burst size of λtrp17E^lNNnin were made in experiment III as shown. One determination each of the trpE^r containing phages 3E^r, 5E^r and 8E^r was made. Superscripts 'l' or 'r' refer to direction of transcription of the trp fragment.

Figure 4-1 Burst sizes of λNNnin phages on W3350

The average burst sizes of the λNNnin versions of the *in vitro* recombinant λ*trp* and control λ*bio* transducing phages, measured as described in the text, are represented on the linear scale shown : 0 - 110 pfu produced per singly infected *sup*^{o} host cell.

The average burst-size of λ*c*I857 itself, with its wild type N-gene and N-control determined under the same conditions was 108 pfu.

Those phages which showed the presence of a *chi*-like site in the qualitative plaque size test are bracketed together as (+) and those which failed to show this response are bracketed together as (-).

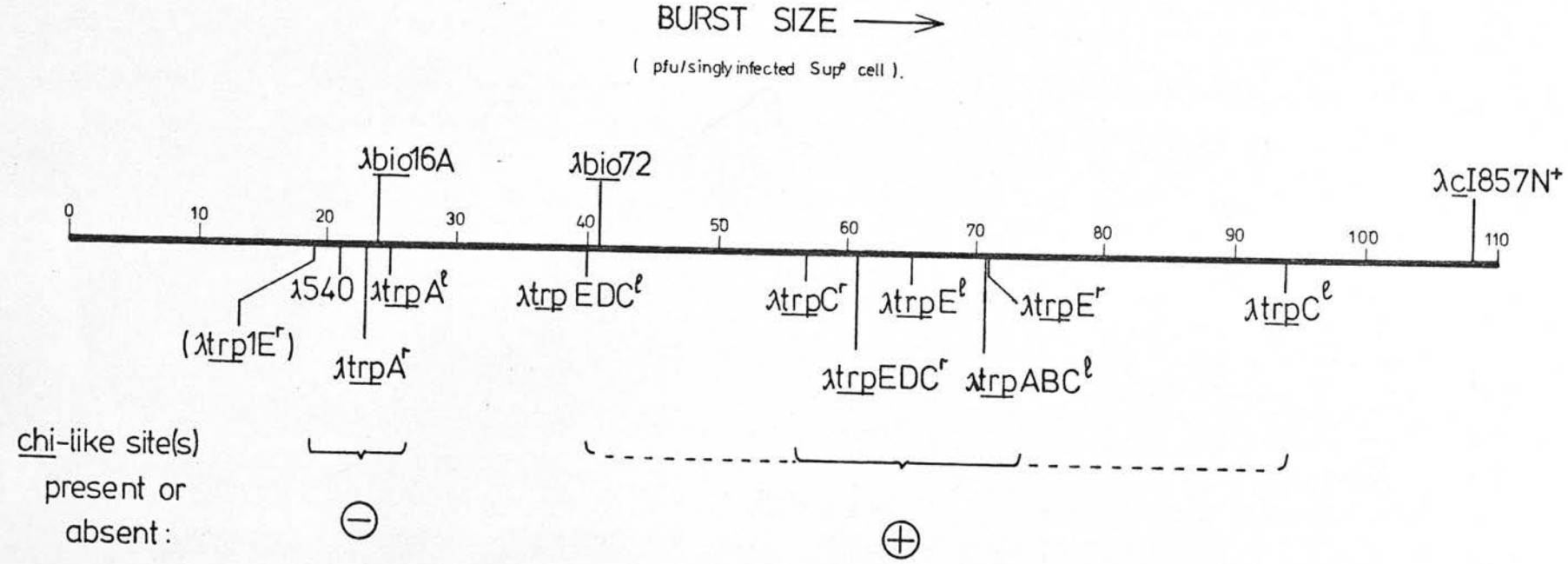


FIGURE 4-1 Burst sizes of the λNNnin derivative phages on W3350.

complementation tests and recombination with Ø80pt190 (Hopkins, personal communication). Since it is difficult to imagine a selective pressure for loss of the chi-like site in the trpE fragment (especially whilst maintaining trpE function), the most likely explanation seems to be that two or more HindIII fragments were concomitantly cloned in the λ540 vector in λtrp1E^{r}, the DNA excess lowering its burst size, as in the case of λbio16A. The direct demonstration of this by analysis of its HindIII digestion products has not been undertaken, but by analogy with λbio16A, it might be predicted that the extra fragment or fragments must together amount to more than 5% λ^{+} length and probably nearer 9% λ^{+} length to cause such a severe reduction in its burst size.

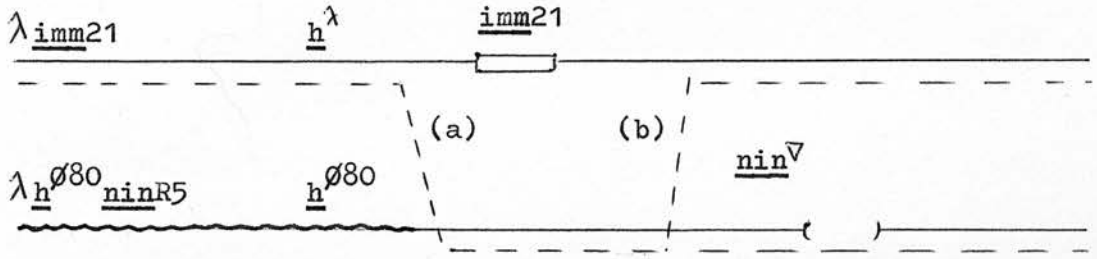
It seems fair to conclude two things from these results: first that the trpE and trpC : HindIII fragments each carry a chi-like site, whereas the trpA : HindIII fragment does not. Second, that the presence of the trpA : HindIII fragment in the λ trpABC^{1} phage does not moderate the activity of the site in the trpC : HindIII fragment, nor does the presence of two chi-like sites in the same phage (as in λ trpEDC) appear to lead to any cumulative effects on plaque morphology or burst size.

As can be seen from Figure 4-1, the burst size is not affected to any significant extent (± 5%) by the orientation of the trpA or trpE fragments. The values for the two orientations of trpC and also for trpEDC, however, are very

different: ($EDC^l < EDC^r$; $C^l > C^r$), but these latter findings are very difficult to interpret, especially as the trpE fragment alone does not confer an orientation dependent increase in burst size, and the optimum orientation of trpC appears to change depending on whether the trpE fragment is present in tandem with it.

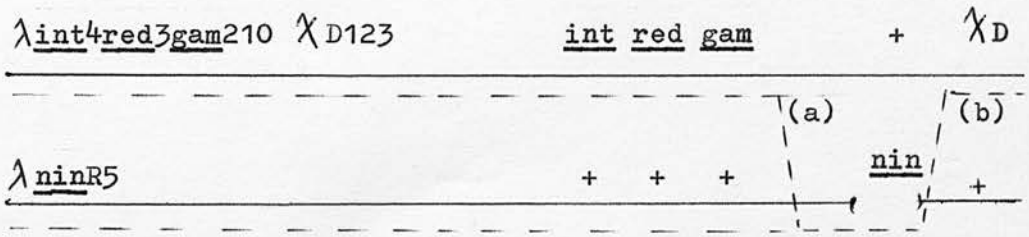
(b) Growth of λ nin phages on groN785

The control phages for these experiments were λ ninR5, which like wild-type lambda does not carry a χ site, and λ ninR5 χ D123 carrying a standard and well characterised χ -mutation (Lam et al. 1974). The ninR5 deletion was isolated in the following cross:



Recombinants were selected on C600 (λ imm21) and purified on C600. Isolates were checked for ninR5 by spot-testing on groN785 with 10^6 pfu per spot. λ nin, from cross-over (a) shown above, will grow under these conditions despite the absence of a chi site, whereas λ^+, from the double event (a) and (b) cannot grow. All isolates tested were of the λ nin type, and one was used as the negative control in the chi-tests described below.

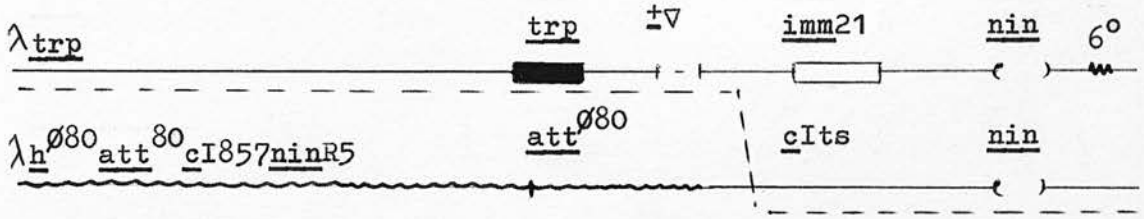
The χ D123 mutation was crossed onto λ ninR5 as follows:



Recombinants were selected as large plaques on groN785 (the nin parent makes tiny plaques and the nin$^+$ parent cannot grow at all on this host) and purified by single plaque isolation on C600. Control experiments showed that λ ninR5 alone gave rise to large plaques on the selective host (by mutation) at a frequency 200-fold lower than in the phage cross (by recombination), thus giving assurance that the χ D123 allele, and not a new χ mutation, had indeed been selected.

The desired recombinant is from event (b) shown above, but the selection would not discriminate between this and the product of the double event (a) and (b) : λ int$^-$ red$^-$ gam$^-$ nin χ D. However, the isolates all showed the Feb$^+$ phenotype, by normal growth on a sup^o polA strain and are thus red$^+$ gam$^+$. (The int mutation is not relevant to this work and was not tested.) One such recombinant was used as the positive control in the chi-tests described below.

Phages which carry the immunity region of phage 21 grow on groN785 even in the absence of a nin mutation and do not respond to the presence of a chi site. (See next section of this chapter.) For this reason the phage 21 immunity of the in vitro recombinant λ trp transducing phages was exchanged for that of lambda in crosses of the following type:



Some of the earlier crosses were to a phage carrying a spontaneous clear mutation (shown to be *cI*$^-$ by the complementation tests described in Methods : h) instead of the ts allele *cI*857.

Recombinants were selected on C600 (λ *imm*21), purified on C600 and checked for complementation of the *trp* genes carried.

λ540*trp*C$^+$(LD102)$^\nabla$ was constructed from the *trp*C$^+$D$^+$E$^+$ derivative by the method described in Chapter 2 (o), inducing a λ *trp*CDE prophage in an *att*λ$^\nabla$ *trp*$^\nabla$ LD102 strain and screening for TrpC$^+$D$^-$E$^-$ phages in the lysate. The λ *trp*A$^+$B$^+$C$^+$(LD102)$^\nabla$ was constructed by Dr. W.J. Brammar.

The *trp* deletions LD102, ED24 and D1 were transferred to the λ722*trp*-transducing phage by growth of the original *trp*A$^+$(BE9) derivative on host strains containing the required *trp* deletions and direct selection of the TrpB$^+$ phages in the resulting phage stocks as Indole$^+$ plaques on ACH agar supplemented with 2ug/ml indole; these new *trp*-deletion derivatives arose at a frequency of 10^{-3} per pfu in the lysates and were selected and propagated on a (*ton*B-*trp*) deletion host with which they have no homology for genetic exchange.

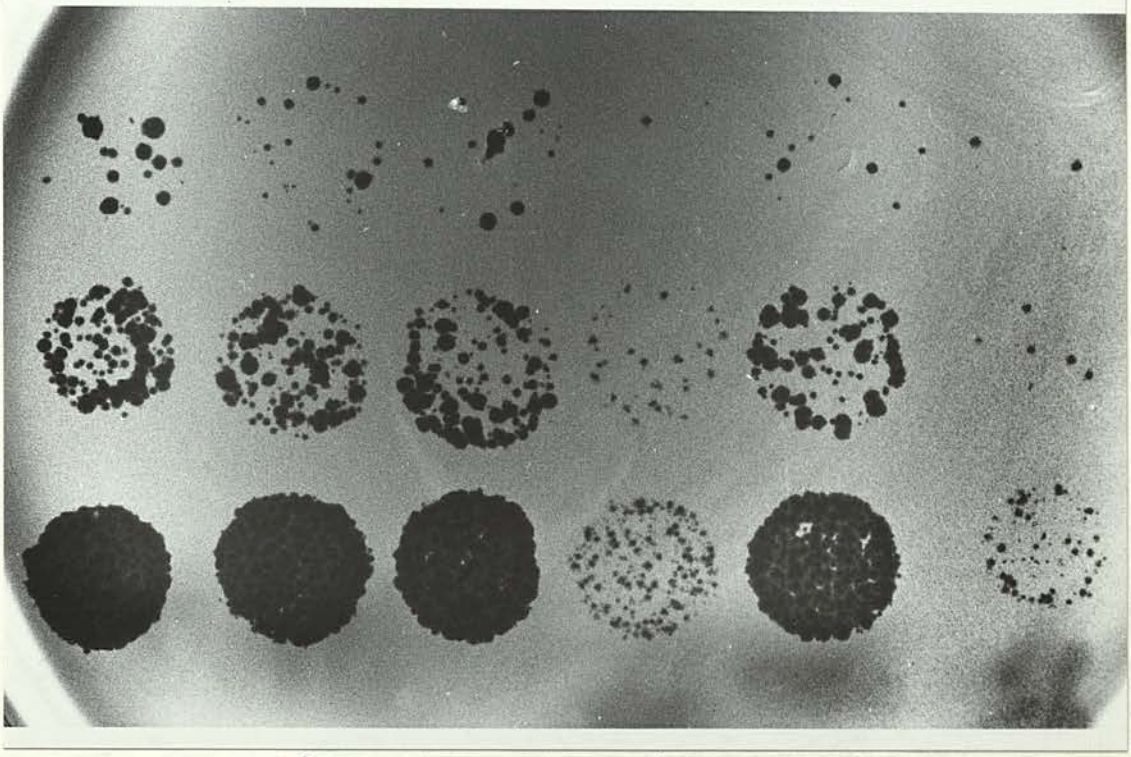
These phages were then used in spot-tests on *gro*N785 and C600 in a search for *chi*-like sites carried by the bacterial DNA they contain. The presence of a *chi* site enhances the growth of these phages on *gro*N785 (in which the *red* and *gam* genes are not expressed) without affecting their normal growth on C600, as shown by the control phages λ *nin* vs. λ *nin*χ D123. Plates 17, 18 and 19 show the results of such qualitative tests.

Plate 17 Tests for chi-like sites in λ540trp phages

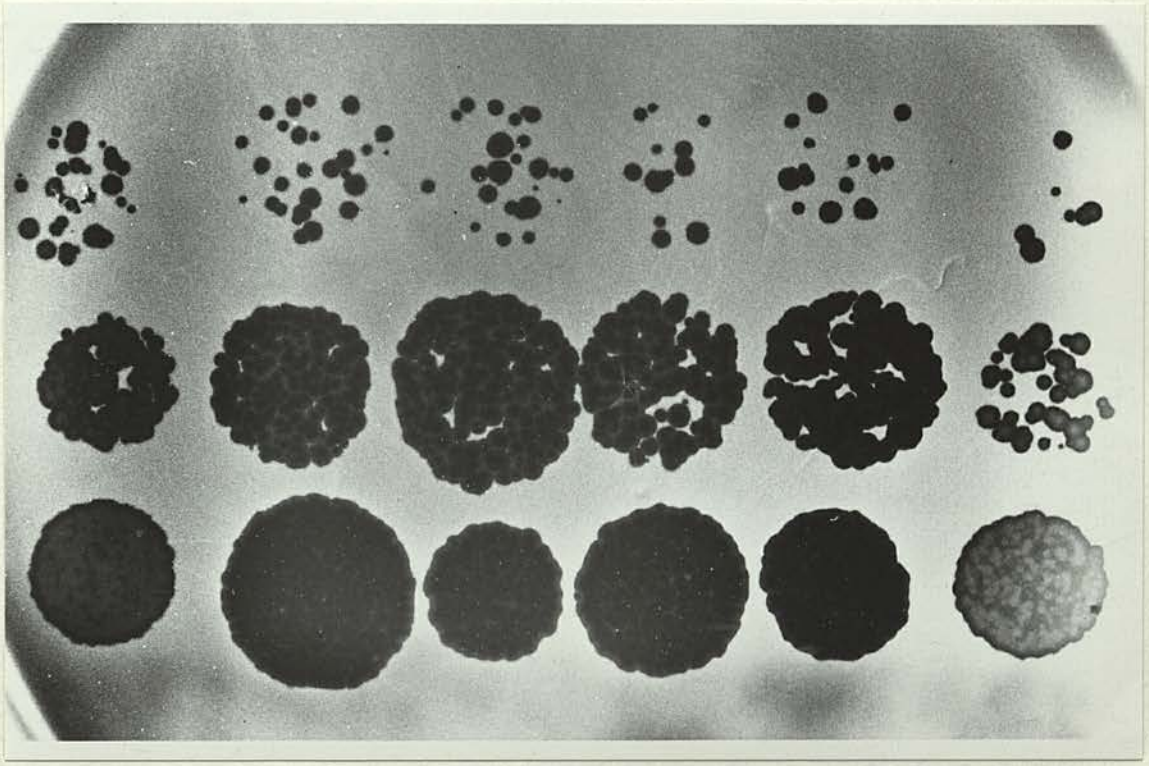
Aliquots (approximately 0.01 ml) of each of three ten-fold serial dilutions of the immλ test phages were spotted first onto plates seeded with groN785 to test for chi-like sites (Upper panel); and then onto C600 to compare phage titres (Lower panel).

Test phages			Chi-like activity
Column	a.	λninR5 χ_{D123} (Control)	+
	b.	λ540trpCDE	+
	c.	λ540trpC	+
	d.	λ540trpABC(LD102)$^{\nabla}$	-
	e.	λ540trpABC	+
	f.	λninR5 (Control)	-

Plate 17



a b c d e f



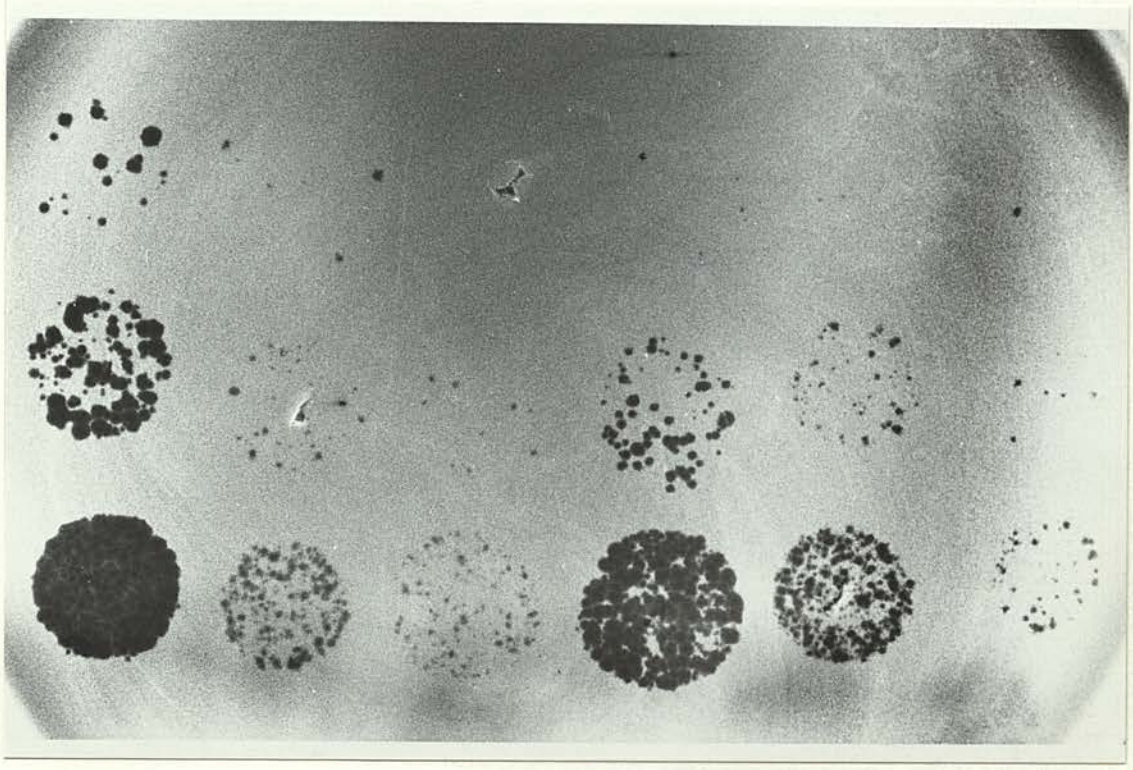
a b c d e f

Plate 18 Further tests for chi-like sites in λ540trp phages

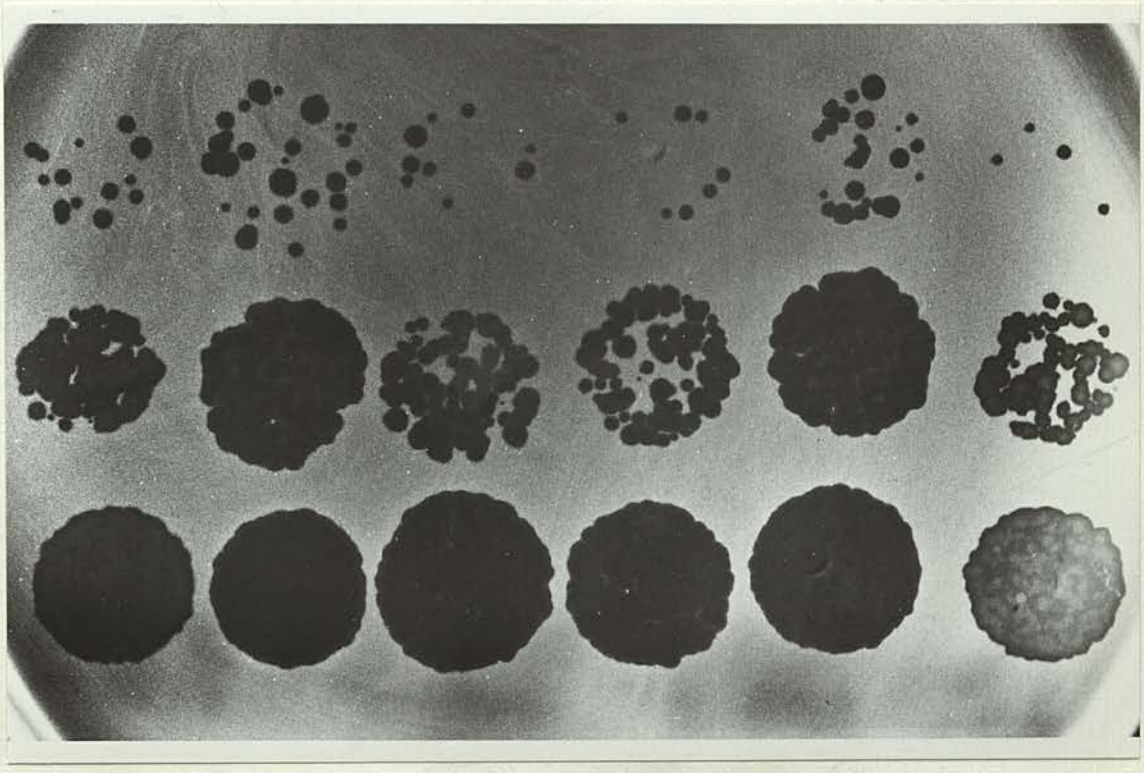
Phage morphology and plaque titre were determined, as before, by spotting 0.01 ml of each of the ten-fold serial dilutions onto groN785 (Upper panel) and then onto C600 (Lower panel).

Test phages			Chi-like activity
Column	a.	λninR5 χ_{D123} (Control)	+
	b.	λ540trpA	-
	c.	λ540trpC(LD102)$^{\nabla}$	-
	d.	λ540trpE	+
	e.	λ722trpA(BE9)$^{\nabla}$	(-)
	f.	λninR5 (Control)	-

Plate 18



a b c d e f



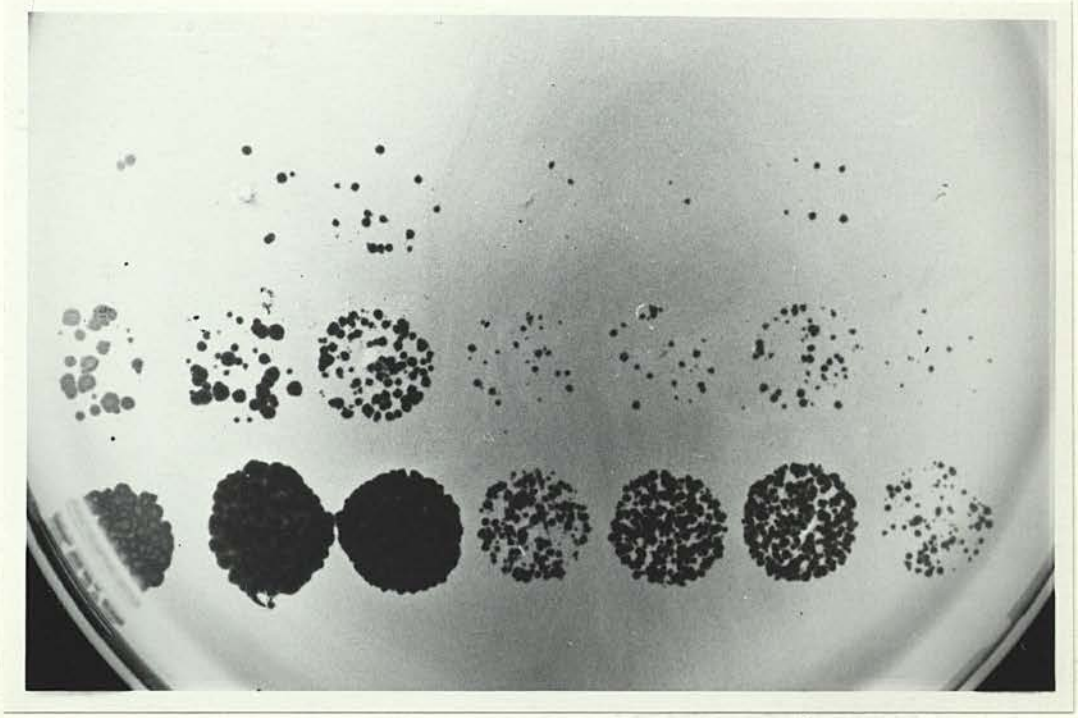
a b c d e f

Plate 19 Tests for chi-like sites in λ722trp phages

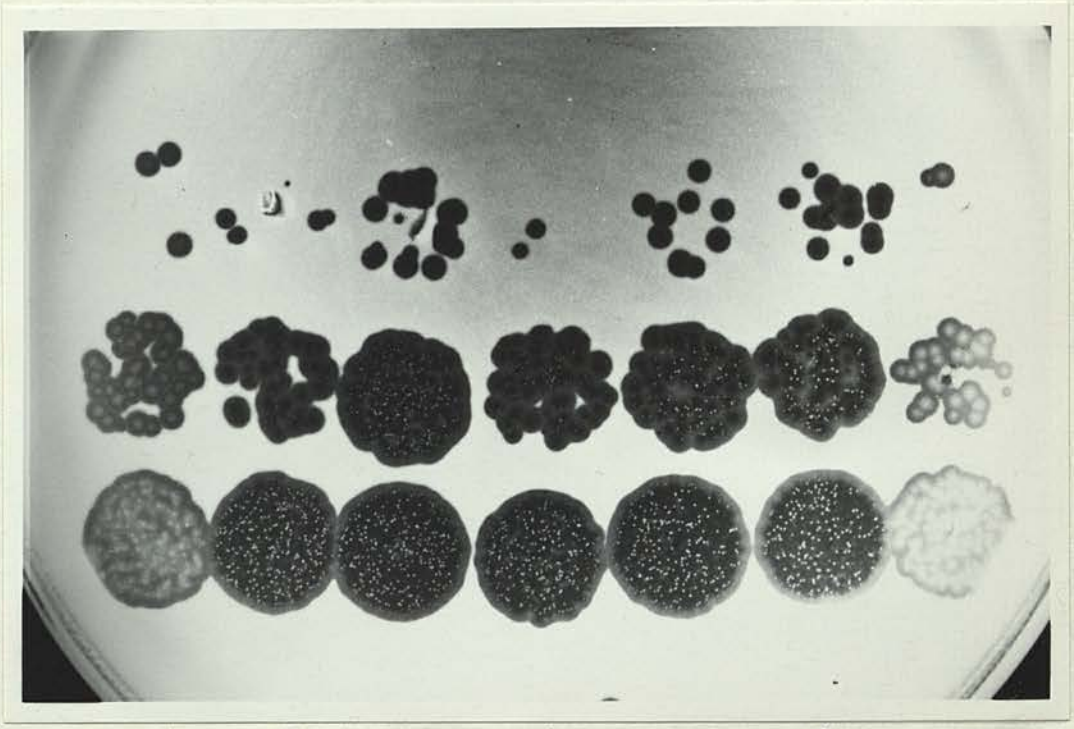
Chi-like sites were determined from spot tests of ten-fold serial dilutions of the phages onto groN785 (Upper panel), and the phage titres were compared from spots of the same dilutions on C600 (Lower panel).

Test phages			Chi-like activity
Column	a.	λninR5 χ_{D123} (Control)	+
	b.	λ540trpCDE	+
	c.	λ722trp^{+}ABCDE	+
	d.	λ722trpABC(D1)$^{\nabla}$	-
	e.	λ722trpABC(ED24)$^{\nabla}$	-
	f.	λ722trpA(BE9)$^{\nabla}$	-
	g.	λninR5 (Control)	-

Plate 19



a b c d e f g



a b c d e f g

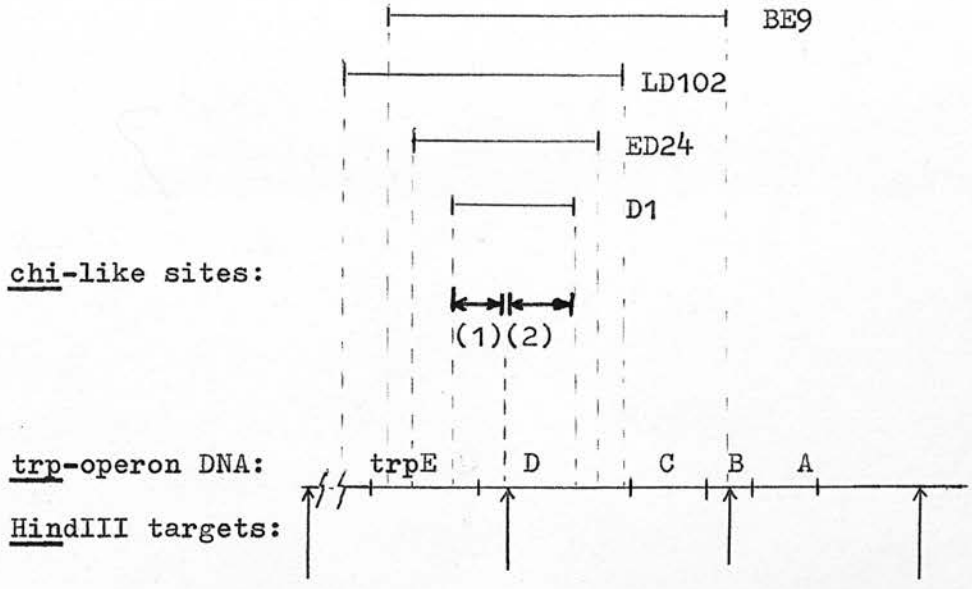
All the vector 540 derivatives used were of the trp(1) series, whilst those of vector 722 were of the trp(r) series, though the evidence presented in the previous section of this chapter suggests that orientation of the trp-fragments is not important for this manifestation of their chi-like activities. As with the previous assay, the trpC$^+$ and trpE$^+$: HindIII fragments show a chi-like activity, whereas the trpA$^+$ fragment does not (Plate 17c, Plate 18b and d). Thus there are at least two chi-like sites within the bacterial DNA fragments tested.

Interestingly, those trp-transducing phages which carry the trpLD102 deletion (Plate 17d; 18d) lose both (or all) of their chi-like sites along with the deleted DNA. Since this deletion overlaps the HindIII target in trpD (See Figure 3-3, mapB) at least one site must lie on each side of this restriction target and within the region covered by LD102. Thus the detected chi-like sites must reside within the trp operon DNA.

The original λ vector722 derivative : λtrpA$^+$(BE9)$^\nabla$ (Plate 18e) shows a plaque size on the groN host which is intermediate between those of λnin and λnin χ D. To test whether this is the positive or negative response of the vector, the trp$^+$ version, which must carry at least two chi-like sites and therefore must elicit a positive response, was constructed. This phage : λ722trp(ABCDE)$^+$, was selected after marker rescue from a trp$^+$ host exactly as done before with the trp internal deletions.

Plate 19 shows the results of comparisons between the λ722trp variants and λ540trpCDE along with the control phages λnin and

λ*nin*χD. It can be seen at once that the positive response is similar in all three cases: λ *nin*χD; λ540*trp*CDE and λ722*trp*ABCDE. The λ722*trp* variants carrying the *trp* deletions D1, ED24 and LD102 all form plaques on the *gro*N host intermediate in size between those of λ722*trp*$^{+}$ and λ*nin*, suggesting that this is the negative response of these λ722 derivatives. Now, the *trp*LD102 deletion has already been shown to remove all the *chi*-like sites in the *trp*-operon DNA, confirming that this is the **response** of the λ722 vector phage in the absence of a *chi*-like site, and defining the position of the *chi*-like sites in the *trp* operon more accurately: See Figure 1-1 for a map of the *trp* deletions.



Thus, the two *chi*-like sites detected in the *E.coli trp* operon DNA must lie under the *trp*D1 deletion, one on either side of the *Hind*III restriction target in *trp*D.

Chi-like sites in substitutions from lambdoid phages

The lambda genome has been shown to be free of chi sites, though they can be readily acquired by mutation (Henderson & Weil, 1974), and it is of interest to know whether chi sites occur naturally in the DNAs of other lamboid phages. The most convenient assay available is the growth of λnin carrying substitutions from these phages, on groN785 as described in the previous section. It is important to remember that the groN host can only be relied upon to block the function of the N protein of lambda itself (Georgeopoulos, 1971; and see discussion : Chapter 6), so that any substitution tested must leave this gene intact.

Substitutions from Ø80 and phage 434 were tested with the results shown in table IV; W1485 was used as the permissive host for the comparison of phage titres, since C600 is resistant to $\lambda\, h^{\emptyset 80}$.

The genomes of all the phages listed in table IV are between 80% and 100% lambda wild-type length and so any growth defect on the groN strain should not be due to a deficit or excess of DNA. (See discussion : Chapter 6).

Phages carrying either the $h^{\emptyset 80}$ or $att^{\emptyset 80}$ substitutions, or both, grow well on groN785, indicating the presence of at least one chi-like site in each of these regions of the Ø80 genome. The small $shn6^{\circ}$ substitution from the Q gene of Ø80 on the other hand, does not contain a chi site, nor does the immunity substitution from phage 434. The slightly enhanced growth of $\lambda\, b\nabla$ imm434nin (line 7, table IV), over that of λ nin is not due to

Table IV

Tests for chi-like sites in substitutions from Ø80 and phage 434

Phage	Substitution	Growth on		chi-like site
		W1485	groN785	
λ nin (control)	-	+ + +	+ - -	-
λ nin χ D123 (control)	-	+ + +	+ + +	+
λ $h^{Ø80}$ $att^{Ø80}$ cI857 nin	$h^{Ø80}$ $att^{Ø80}$	+ + +	+ + +	+
λ $h^{Ø80}$ cI∇ nin shn6°	$h^{Ø80}$, shn6°	+ + +	+ + +	+
λ srI(1-2)∇ cI∇ nin shn6°	shn6°	+ + +	+ - -	-
λ $att^{Ø80}$ Nam7am53nin	$att^{Ø80}$	+ + +	+ + +	+
λ b∇ imm434nin	imm434	+ + +	+ + -	-
λ $att^{Ø80}$ imm434nin	$att^{Ø80}$ imm^{434}	+ + +	+ + +	+

The nin mutation used was the deletion ninR5.

The $h^{Ø80}$ substitution replaces the left arm of lambda with Ø80 material of 40% lambda length; $att^{Ø80}$ is carried by a DNA segment contiguous with the $h^{Ø80}$ substitution and of 10% lambda length, (Fiandt et al., 1971).

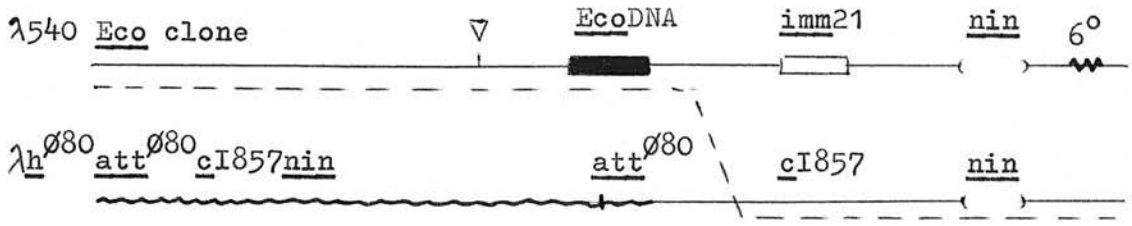
shn6° is a small (< 3%) substitution from Ø80 and includes part of the Q gene (Described in Blattner et al., 1977). The immunity region of phage 434 is carried by a DNA segment of 6.5% lambda length (Simon et al., 1971).

a chi-like site, since its vigour is markedly increased by an additional att$^{\phi 80}$ substitution (line 8), whereas cumulative effects are not seen in other groN tests. (Compare lines 3 and 6).

When grown on the groN host λimm21 was found to give small plaques comparable in size to those of λnin, whereas λimm21nin was found to give large healthy plaques like those of λnin χ D123; also, λ540 (λsrI(1-2)$^{\nabla}$ imm21nin6°) and λ540trpC produced indistinguishable large healthy plaques. Thus, the known chi-like site in trpC does not visibly affect the growth of λimm21nin, and therefore phages carrying imm21 cannot be directly tested for chi sites by this method.

Chi-like sites in cloned fragments of E.coli DNA

E.coli DNA fragments from digests with the restriction enzymes EcoRI and HindIII have been cloned into lambda vectors and several individual in vitro recombinants transducing identified genes have been selected from them (Borck et al., 1976). These clones were used in a limited survey of chi-like sites within the E.coli genome. All the recombinants carry the ninR5 deletion. The EcoRI clones have the immunity of lambda and were screened directly by parallel spot-tests on groN785 and C600 as before. The imm21 substitution carried by the HindIII clones was exchanged for that of lambda in phage crosses of the following type:



The desired recombinants were selected on C600 (λ imm21) and purified by single plaque isolation on C600.

Each clone was checked for the particular phenotype conferred by the E.coli gene(s) it transduced (as described in Chapter 2, section 6) before performing the spot-tests on groN785 & C600. The results of these tests are shown in Table V.

The phages λplac5 (Ippen et al., 1971) and λpgal8 (Feiss et al., 1972; Chow et al., 1974), and λpbio72 (Hradecna & Szybalski, 1969), which were constructed by in vivo techniques were also used in this survey; the results of tests with their ninR5 derivatives are shown in Table V.

The phages λnin and λnin χD123 were run in parallel with each experiment and titres compared on C600 as before. The unsubstituted vector phages from which the clones were derived (or their lambda immunity derivatives) always gave a small-plaque response on groN785 showing that they lack a chi-site.

Only in the case of λcysB does the size of the genome of the transducing phage approach the limits of viability and hence prejudice the result of the groN-test; for this reason the assignment for the E.coli cysB fragment must remain tentative.

Table V: Tests for chi-like sites in E.coli DNA fragments

(a)

EcoRI clones:	E.coli fragment size (Kb)	plaque size on groN785	chi-like site
1. λ cysB	3.5-4.0	small	(-)
2. λ hsd_KMS	12.0	large	+
3. λ lig	9.5	small	-
4. λ D13	4.5	large	+
5. λ supD		large	+
6. λ supE		small	-
7. λ supF		small	-

(b)

HindIII clones:	E.coli fragment size (Kb)	plaque size on groN785	chi-like site
1. λ hisG	5.5	small	-
2. λ hisBHAF	4.5	small	-
3. λ thyA		large	+
4. λ tnaA	7.0	small	-
5. λ supF	8.0	small	-
6. λ polA	5.0	small	-
7. λ glnA	7.0	small	-
8. λ trpA	3.0	small	-
9. λ trpC	2.9	large	+
10. λ trpE	6.2	large	+

(c)

Other transducing phages:	E.coli fragment size (Kb)	plaque size on groN785	chi-like site
1. λ plac5cI857nin	5.0	small	-
2. λ pgal8nin	5.1	small	-
3. λ pbio72NNnin	4.2	large	+

The λ*lig* *lop*-11 described in Borck *et al.* (1976) was cloned from the ligase over-producing strain, *lop*-11, and gave a positive result in tests like those shown in Table V. This phage differs from the λ*lig* tested previously, only in the orientation of the *lig* fragment in the phage, and in the *lig* promoter (K. Borck, personal communication). Thus it would seem that either the *lop* mutation creates a *chi*-like site, or the fusion of the *lig* fragment with the vector DNA creates such a site when the fragment is present in one orientation, but not in the other. The final alternative is that some other structural change, not related to *lop*-11, and which has not been detected results in the acquisition of a *chi*-like site.

Phage D13 is the intermediate (cited in Borck *et al.*, 1976) carrying an uncharacterised fragment of *E.coli* DNA of 9% lambda length, from which λ*lig* *lop*-11 were produced.

An extrapolation can be made from the above data giving an estimate of the frequency of *chi*-sites in the *E.coli* genome (assuming that the sites assigned are indeed *chi*-sites and not sites of a different nature conferring the same phenotype):

E.coli clones	Number tested	*E.coli* DNA surveyed	*chi*-sites found	avg. separation of *chi*-sites
EcoRI fragments	7	ca 52.5Kb	3	17.5Kb
HindIII fragments	10	ca 55Kb	3	18.3Kb
'*in vivo*' phages	3	14.3Kb	1	14.3Kb

Thus, the estimate which emerges is that the E.coli genome containing about 4,000Kb (Klotz & Zimm, 1972) probably contains 200 - 250 chi sites, or one per 12 - 15 genes.

CHAPTER 5

LAMBDA AS A PLASMID

Lambda can propagate as a plasmid when its N gene is inactive (Signer, 1969; Lieb, 1970); and a small segment of the lambda genome can exist independently as a plasmid: λ*dv*, present in 50 - 80 copies per cell (Matsubara & Kaiser, 1968; Berg, 1974; Chow *et al.*, 1974). The classical λ*dv*-1, which contains only 15% of the lambda genome, has been used as a cloning vehicle for foreign DNA (Mukai *et al.*, 1976).

It seems reasonable to suppose that λNN⁻ which exhibits similar properties to λ*dv*'s, but maintains the entire lambda genome, could also be used as a receptor molecule for cloning experiments - with the added advantage that recovered recombinant DNA could be propagated as a plaque-forming phage on hosts in which its N gene was functional. Genetic analysis of plaque-forming lambda transducing phages is generally technically easier than that of plasmids: transfer to different hosts prior to recombination or complementation tests being a trivial procedure.

Thus, foreign DNA fragments (regardless of size) could be cloned into an appropriate restriction target in a λNN*am* vector and propagated as a plasmid and recombinants selected. Covalently closed circular (CCC) DNA recovered from this transformed strain could then be used to transfect a *sup*$^+$ host, demanding plaques and hence selecting for deletions where necessary, to bring the transducing phage into the maturable size range. Analysis of a series of such phages, carrying deletions of different parts of the cloned fragment, would allow a genetic map of the original to be built up.

The five EcoRI targets of lambda have been manipulated to generate vector genomes with one, or two targets for use as cloning vehicles by insertion or replacement, respectively (Murray & Murray, 1974; Murray et al., 1977). The NNam nin$^+$ versions of these receptors would fulfil most of the requirements of the proposed vector, but a selection for transformed cells bearing lambda-plasmids was also needed. Two selective procedures were investigated; one, the Lac$^+$ phenotype conferred on lacz$^-$sup^o hosts by λplac5 (Ippen et al., 1971) and the other, ampicillin resistance mediated by the E.coli TEM β-lactamase of the transposon TnA (Heffron et al., 1977).

A lacz$^-$sup^o strain with a high competence for DNA uptake was required, and this, along with its supF and recA derivatives, was constructed from SA291trp$^\nabla$ LD102 : a known highly competent strain.

Construction of competent strains A125, A126 and A129

The T6-receptor gene is closely linked to lac in the E.coli chromosome, and was used as the coselection for the P1-transduction of the lacz deletion M15. A T6-resistant derivative of the laci3laczM15 donor, XA21 was selected and a high-titre lysate of P1kc grown on this strain. The lysate was then used to transduce SA291trpLD102 to T6-resistance, and transductants selected on lactose-MacConkey agar spread with 10^{10} pfu T6. About three to five percent of the surviving colonies were white (Lac$^-$) and when tested were able to complement the lacz fragment of phage λ626 (Murray et al., 1977) by alpha-omega complementation (Ullman,

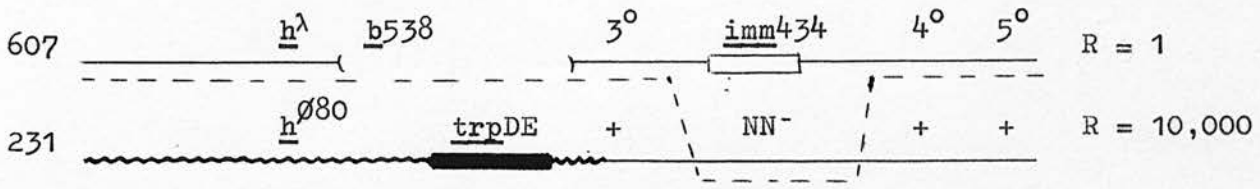
Jacob & Monod, 1967) to give red, Lac$^+$ plaques on lactose-MacConkey agar. One such transductant was purified and named A125.

The supF derivative : A126, was constructed by P1-transduction of A125 to Trp$^+$ using a high-titre lysate of P1kc grown on Ymel (a supF trp$^+$ strain), and testing the transductants for ability to support the growth of λSam7 and λNam7am53. About thirty percent of the Trp$^+$ progeny were able to suppress both these mutants and were judged to have acquired the supF genotype.

The recA56 allele was introduced into A125 by Hfr mating with JC5088 and selecting His$^+$ ex-conjugants, as described in Methods (m). Purified His$^+$ colonies were replicated to minimal agar supplemented with biotin and with biotin and tryptophan; Trp$^-$ colonies (retaining the trpLD102 deletion) were picked and scored for recA by testing their ability to support the growth of λbio-1 and of λ$^+$ (the Fec phenotype). All were found to give small plaques with the wild-type, but gave none with the bio phage (Fec$^-$) and were taken to be recA$^-$. One of these colonies was picked and named A129.

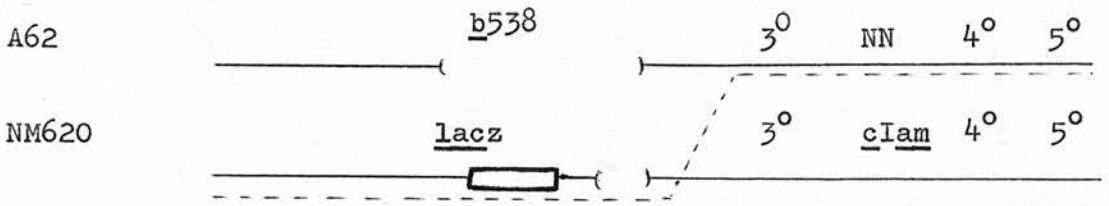
Construction of lambda-plasmid vectors

The λsrI3°4°5° mutations of the RI restriction targets in lambda were derived from λ607 and the Nam7am53 mutations rescued from λtrp51NN$^-$ (WB231) in the following phage cross:



The progeny were grown for one cycle on 5K/RI to enrich for phages lacking RI restriction targets (moi = 0.1 - 0.5, at a cell density of 10^6/ml in L-broth, grown at 37° with good aeration for 75 mins, and the lysate sterilised with several drops of chloroform). Recombinants were selected on C600 (λ imm434) and purified on C600, and their RI-restriction ratios measured by spot-titration on 5K and 5K/RI. A recombinant with a restriction ratio of unity was checked for NNam by its reversion frequency (found to be ca. 10^{-9} per pfu by growth on the sup° hosts W3350 and A125) and was used as the donor of the right arm (srI3°NNam4°5°) for the lambda-plasmid vectors.

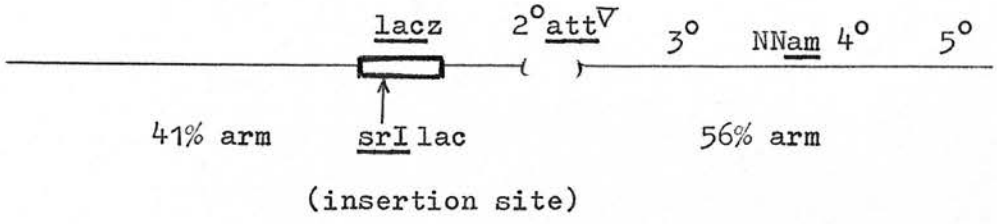
The λ plac5NN⁻ vector was constructed by the following cross:



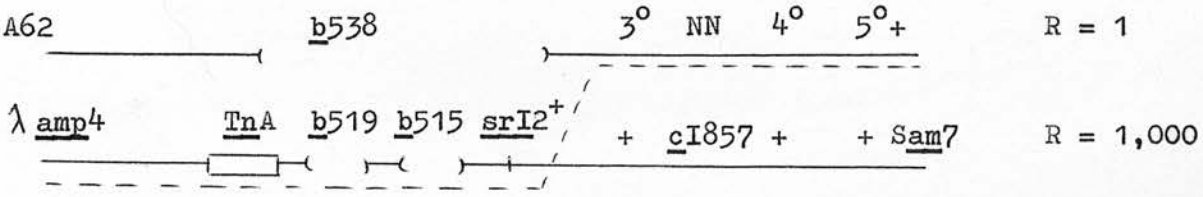
Progeny were grown on A126 (the supF host) on BBL-XG agar. Phages carrying plac5 make blue plaques on this medium, and λ Nam7am53 produces 'cocarde' plaques with a small central turbid ring (due to undersupply of active N-product for cI expression on a supF host : see Reichardt, 1975). Thus, the desired recombinant was sought amongst the 'cocarde' blue plaques; candidates were purified and tested for plaque-formation on A125 (sup°) and their RI-restriction ratios determined on 5K and 5K/RI. Of those picked only a few were λ plac5NN phages as judged by their low

reversion frequency (10^{-9}) on a sup° host and RI-restriction ratio of 5; one such isolate was kept as the λ lac5NN vector.

λ lacNN:64 R = 5



TnA, specifying the E.coli penicillinase, has been transposed to various positions in the b2 region of the lambda chromosome (Hayes, 1976), and one of these phages : λ amp4, was used as the donor of TnA in the following phage cross:

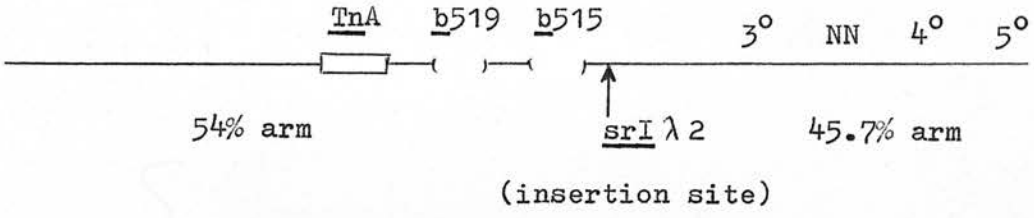


The progeny were grown for one cycle on 5K/RI to enrich for phages with few RI targets as before, and the resulting lysate plated on C600 (which does not suppress the Sam7 mutation and hence does not allow these phages to form a plaque) at 37°C.

Well isolated single turbid plaques were then picked and spotted onto duplicate plates overlaid with a lawn of C600, one of BBL agar and the other of BBL-PVA agar. After overnight incubation the spots formed turbid areas of lysis, and the BBL-PVA agar plate was stained for penicillinase as described in

Methods (1). The recombinants carrying TnA were recognised by the decolourised halos surrounding their plaques and each was recovered from the duplicate plate, tested for plaque-formation on A125 and its RI-restriction ratio determined by accurate titration on 5K and 5K/RI. Of the λ TnA:NN recombinants screened in this way, most had an RI-restriction ratio of ca. 50; denoting the presence of more than one RI-target, but a few had a ratio of only 2.5, characteristic of srI λ 2 alone (Murray & Murray, 1974). One of these latter recombinants was picked as the λ ampNN vector.

λ ampNN:63



Transduction frequencies and stability of λ NN-plasmids

The frequency at which a sup° cell, singly infected with λ NN⁻ forms a plasmid-bearing clone was examined for each of the proposed λ NN-vectors and compared with that of λ trp51NN. The transduction frequency was expressed as the number of pfu on A126 divided by the number of clones selected on A125 in parallel experiments.

Infecting genome	Selection	Selective Medium	Transformation frequency
λ trp51NN	Trp^+	minimal + ACH agar	0.33, 0.48
λ lacNN:64	Lac^+	lac.+his,trp,bio.	0.12, 0.18
λ ampNN:63	Amp^R	L-agar + ampicillin	0.29, 0.37

Amino-acid supplements were added to a final concentration of 20 ug/ml; lactose at 0.2%; and ampicillin at 20 ug/ml.

The Amp^R colonies grew up in 12 - 18 hours and the Trp^+ clones in about 36 hours, but the Lac^+ colonies grew very slowly, giving only tiny colonies on the selective medium even after three days. The low transformation frequency observed for the λ *lac*NN:64 plasmid is probably because some of the Lac^+ colonies were too small to see.

One of the transformed colonies of each type was picked and grown in liquid selective medium (10 ml, with the same supplements as shown above), and tested for immunity to superinfection by λ *c* and λ *vir*; the supernatant of each culture was titred for free phage on A126.

Lambda-Plasmid Selection:	λ *trp*51NN Trp^+	λ *lac*NN:64 Lac^+	λ *amp*NN:63 Amp^R
eop of λ *c*	10^{-6}	10^{-6}	0.5
eop of λ *vir*	1.0	1.0	1.0
free pfu/ml	10^3	10^3	10^5
$Trp^-/Lac^-/Amp^S$ cells in culture	Not done	0 / 60	76 / 306

From the above data it can be seen that the Amp^R phenotype was readily lost in liquid culture: this may be because of depletion of ampicillin by liberated penicillinase during the

18 hour growth period, but this hypothesis was not tested. However, an Amp^R colony grown on solid selective medium contained few, if any, Amp^S cells (<9% 97 colonies from a dilution of a colony selected on L-agar and 20 ug/ml ampicillin, as judged by replica plating tests).

Thus, whilst the Ampicillin-resistance selection for λ*amp*NN:63 appears effective, and the plasmid bearing clones stable (at least on solid selective medium), the selection for clones containing λ*lac*NN:64 is weak and very slow. Only the λ*amp*NN:63 vector was used in subsequent trial cloning experiments.

Restriction & ligation experiments with the λampNN:63 plasmid

DNA was prepared from λ*amp*NN:63 grown in the lytic mode, on C600, and also from *E.coli* W3110. The DNAs of λ806 or λ461 were used as convenient competence controls in transfection experiments, since they form plaques on all the hosts used. Such experiments gave the following competence levels routinely:

Transfection host	λ806 or λ461 DNA	λampNN:63 DNA
A125 (*sup*°)	2-2.6 x 10^6 pfu/ug	1-1.5 x 10^6 transfmts/ug
A126 (*supF*)	1-1.5 x 10^6 pfu/ug	1-1.5 x 10^6 pfu/ug
A129 (*recA*)	1-1.5 x 10^6 pfu/ug	0.8-1.2 x 10^6 transfmts/ug

0.1-1.0 ng (10^{-9}-10^{-10}gm) intact phage DNA was incubated with 0.2 ml calcium-treated cells for each competence assay, according to the standard procedure described in Methods (y), and plated either for plaques on BBL agar, or for Amp^R colonies on L-agar and 20 ug/ml ampicillin, as appropriate.

The procedure adopted for trial cloning experiments was as follows: 2-5 ug λampNN:63 DNA and an equal weight of E.coli W3110 DNA were separately restricted with EcoRI as described in Methods : 2(t). The best results were obtained when restriction was performed in the presence of 0.1 volumes 'Porton dilution buffer', which contains:

20mM Potassium phosphate, pH 7.4

50mM NaCl

10mM 2-mercapto-ethanol

0.1% Triton-X100

2 mg/ml gelatin

(sterilised by autoclaving before use)

Cutback in transformation efficiency of the lambda plasmid DNA due to restriction was assayed using 10-50 ng restricted DNA, just as for, and in parallel with, intact lambda plasmid DNA, to transform 0.2ml competent cells to ampicillin-resistance. Cutback was generally 50-1000 fold for the single site vector, the lower figures correlating with the most efficient religation in later stages.

Restricted vector and E.coli DNAs were mixed in equal proportions and the ligation reaction carried out as described in Methods : 2(x). Religation was monitored at 1-2 day intervals by transformation of competent cells as before, and where effective, 7-8% of the original transformation efficiency per ug vector DNA was recovered after 2-6 days.

In experiments where recovery was observed after ligation (to 2% and 7%), recombinants containing E.coli W3110 DNA were sought.

Aliquots of ligation mixture containing 1,000-2,000 Amp-transforming units were used to transform 0.2ml aliquots of calcium-treated, competent A125 cells and the mixtures spread ~~onto~~ on selective glucose-minimal plates containing 20 ug/ml ampicillin plus two of the following: tryptophan, histidine and biotin (0.05% ACH was added to the plates lacking tryptophan). Other aliquots were spread onto galactose-or lactose-minimal agar containing all three amino acid supplements, and 20 ug/ml ampicillin. These selective procedures were designed to detect λplasmid recombinants carrying the trp,his,bio,gal and lac regions of the E.coli chromosome, but no such recombinants were selected. Since religation of the vector had been amply demonstrated, the E.coli DNA preparation used was suspect.

Effort was then directed towards cloning the EcoRI:trp operon fragment of λtrp$^+$51 into the lambda plasmid and the above procedures were repeated, but on a 1ug scale. Again, restriction and subsequent ligation were shown to have occurred efficiently (cutback was to 0.2% and recovery to 2% original transformation efficiency) as judged by transformation of A125 to ampicillin resistance. However, the λtrp$^+$51 DNA used was unmodified and sensitive to restriction by an E.coli K strain such as A125; the EcoRI:trp fragment of λtrp$^+$51 carries two K restriction targets (see Chapter 3) and would be expected to be restricted a thousand fold. For this reason, despite a 4-5 fold sacrifice of competence in DNA uptake, the strain ED8639:(803 sup^o $R_K^-M_K^+$,trpBE9∇,trpR$^-$) was used in an attempt to select Amp^{R}Trp$^+$ recombinant clones. Aliquots of the ligation mixture containing 200-300 Amp-transforming

units were spread onto ACH-agar containing 20 ug/ml ampicillin. Several small colonies were found on each plate after 36 hours incubation at 37°C. These clones grew poorly when streaked onto fresh selective medium to purify the recombinants and on the second round of purification did not grow at all. As discussed in Chapter 6, this instability may well be due to the *trpR*$^-$ genotype of the host and a repetition of the experiment using a *trpR*$^+$*trp*$^-$ (non-restricting) host, such as *E.coli* C-1a *trp*AE8, remains to be done.

CHAPTER 6

DISCUSSION

Physical and genetic analysis of λtrp transducing phages

The heteroduplex evidence presented shows that the bacterial DNA carried by λ*trp*$^+$51 is 7.4 ± 0.7 Kb (15.1 ± 1.5% lambda) in length, only slightly longer than the estimated 7.0 Kb minimum extent of the *trp* operon it contains. This demonstrates that it is unlikely to carry any other *E.coli* genes, and indeed it was shown to be unable to transduce the *tonB* gene which lies between the bacterial Ø80-attachment site and the *trp* operon in the host chromosome; thus λ*trp*$^+$51 cannot have been formed by a single reciprocal cross-over event according to the classical Campbell model (Campbell, 1962). The evidence is consistent with the formation of λ*trp*$^+$51 from λ*trp*51 by rescue of the wild type *tonB-trp* segment of the host chromosome, followed by the selection of a secondary *tonB* deletion bringing the genome of the lambda-*trp*$^+$ transducing phage into the viable size range.

The λ*trp*$^+$51/λ*trp*51*trpOE1* heteroduplex shows a 'bubble' of non-homology, the substitution running from the expected map position of *trpOE1* (Brammar *et al.*, 1974) on the left, across the Ø80 material including the *xis* gene and into the lambda-*red* region on the right. This substitution in the phage must have occurred by an illegitimate recombination event, without any homology between the lambda DNA on the one side and the new DNA upstream of *trpE* on the other, and this correlates with the much lower frequency at which it arose compared with the pick-up of *trpLD102* : involving only

legitimate cross-overs in homologous regions. The nature of the chromosomal trpOE1 mutation remains obscure; it could be a deletion, an insertion or a substitution, divorcing the trp control region and a promoter-proximal part of the trpE gene from the rest of the operon, which is then coupled to an efficient unregulated promoter.

The trp operon is excised intact by the EcoRI endonuclease and analysis with this enzyme has been very useful in the determination of the extent of gross structural changes in the operon. The EcoRI : trp fragment from the λtrp51 series, however, also contains the contiguous ø80 and lambda material on each side of the bacterial DNA, showing that the targets for this enzyme flanking the trp region of the E.coli chromosome are not present in these phages.

The data of Chapter 3 lead to an unambiguous map order for the seven HindII & III restriction fragments detected in digests of the trp operon DNA (Figure 3-3, map B). The possibility remains, however, that one or more very small fragments coming from sites less than 150 base pairs apart may have escaped detection. If this turns out to be the case, then the additional site(s) must be cleaved by the HindII enzyme, since the two HindIII targets mapped have been shown to be the only ones present in the trp operon DNA (Hopkins et al., 1976).

Statistical considerations show that HindIII sites would be expected to occur on average once per (ca. 4^6) 4,000 base pairs, whereas HindII sites should have a mean frequency of one per 1,000 base pairs (ca. $4^4.2^2$), bearing in mind the redundancy of the recognition sequence. Thus two HindIII sites and eight HindII sites

would be expected within the 8Kb trp DNA region analysed. In fact, the actual observations show two HindIII sites and six HindII sites.

Using the known position of the HpaI site in the trp-promoter (Bennett et al., 1976) the genetic and physical maps of the trp operon have been aligned and show close agreement, (Figure 3-3, maps A & B). The HindIII site in the trpD gene falls between the D156 and D562 mutations in the alignment and this result is corroborated by genetic evidence (Hopkins et al., 1976). The HindIII site in the trpB gene is known to lie between the B4 and B18 mutations, but only the order of these mutations as determined by deletion mapping (Crawford et al., 1970) is known and no linkage data is available (the order of the trpB mutations shown in Figure 3-3 is correct, but their positions are arbitrary). The present results show a HindIII site in the promoter-proximal half of the trpB gene, which is consistent with its genetic map location.

The restriction data suggest that the HpaI target at the promoter-distal end of the operon falls within the trpA gene, one hundred base pairs from the promoter-proximal end. However, comparison of the amino-acid sequence of the trpA protein (Yanofsky et al., 1967) and the possible amino-acid sequences specified by a HpaI site:

mRNA: 5'-GUU-AAC-3'; G-UUA-ACX- ; XGU-UAA-CXX;

-val-asn- -leu-thr- x'-ochre.

shows that there can be no HpaI targets within the trpA gene. Thus, the mapped target probably lies in the trpB gene, close to trpA, and the inaccuracy in its determined position is probably due to cumulated errors involved in adding together several measured fragment lengths.

Since λ*trp*$^{+}$51 carries only 7.4 ± 0.7 Kb of bacterial DNA, (see earlier discussion) and the sum of the lengths of the *Hind*II & III fragments of the *trp* operon in its digests is 8.1 Kb, the last mapped *Hind*II target, downstream of the *trp*A gene, is probably within the Ø80 material.

The single *Xho*I target observed in the *E.coli* DNA carried by λ*trp*$^{+}$51 and λ540*trp*A was measured to be downstream of the *trp*A gene, but since the *Xho*I fragment is large in each case, so is the possible error in its placing. To test whether there are any positions in the *trp*A gene which are compatible with an *Xho*I target, the *trp*A protein sequence was scanned for possible translation products of the *Xho*I recognition sequence:

*m*RNA:	5'-CUC-GAG-3';	C-UCG-AGX ;	-XCU-CGA-G.
amino acid sequence	-leu-glu-	-ser-ser-	-ser-arg-
		-arg	-pro
			-thr
			-ala

Two such sequences occur: one at residues 48 - 49 (leu-glu) and the other at 178 - 180 (ser-arg-ala). Note that the positions refer to the corrected sequence, with an extra ile-residue at position 37 (cited in Li & Yanofsky, 1972). It has been shown that *trp*A88, a mutation to an amber codon (UAG) at position 49 in the *trp*A protein sequence, does not affect the *Xho*I target (Anilionis & Brammar, unpublished experiments). Also, the *m*RNA sequence at positions 178 - 180 has been deduced (Brammar *et al.*, 1967) as UCA-CGA-GCX : a sequence not recognised by *Xho*I. I conclude that the *Xho*I

target observed in the λtrp phages tested does not reside in the trpA gene.

The analysis of AvaI digests of the λtrp51 series shows two targets in the bacterial DNA downstream of the trpA gene, ca. 450 base pairs apart and one of these has been shown to be cleaved by XhoI. The other site must be shared by the enzyme XmaI, since λtrp$^+$51 contains a single XmaI target in this region (Pouwels, unpublished results) and, like XhoI, the XmaI recognition sequence is a subset of that of AvaI.

The lambda : Ø80 hybrid phage 407.5, from which the λtrp51 series were originally derived, has been shown to retain the EcoRI site 3 in the λred α gene at 65.6% on the standard lambda map, whilst the AvaI site 5 at 65.2% is no longer present and is replaced by Ø80 DNA. This places the exchange between lambda and Ø80 material in the hybrid very accurately within a block of partial sequence homology including the red genes (see Figure 1-2) and indicates that the recombination event producing the hybrid phage was homology dependent.

The present results of the use of deletions of λtrp$^+$51, both well-characterised and newly selected by the pyrophosphate technique, in the elucidation of the physical map of the operon illustrates that a great deal of detailed structural information can be gained by restriction analysis. The knowledge of the precise locations of restriction targets is a necessary prerequisite

to the modern methods of DNA sequencing (Sanger & Coulson, 1975; Gilbert & Maxam, 1977), which can only be used to determine sequences within 100 - 150 bases of the target itself. Thus, nucleic acid sequences of particular regions of interest can only be determined by these techniques after a detailed restriction map has been obtained. In the present case of the trp operon, the mapped HindII and HindIII targets fall within the structural genes and the DNA sequences of regions which flank them may be useful for the analysis of mutational changes causing defects in the tryptophan biosynthetic enzymes.

Figure 3-5 shows the distribution of the XhoI targets in λ vector540 and λ540trpA(r). Since the former has only one site it could be used as an insertion vector for cloning XhoI digestion products of 0 - 12.5 Kb in length, and the latter, which has two targets, is an ideal replacement vector for fragments in the 4 - 19 Kb range. Replacement of the central fragment of λ540trpA(r) by foreign DNA would remove the red and gam genes, allowing such recombinants to be selected directly on C600(P2) by their Spi^- phenotype (Lindahl et al., 1970). It remains to be shown whether the inversion of the central fragment in this phage (putting the red and gam genes under lambda late control) would also generate Spi^- phages: since their expression would be late in the lytic cycle, the presence of the red and gam genes may not interfere with growth on the P2-lysogen, and they may form plaques along with the recombinant phages. Incorporation of DNA into the XhoI site in λ540 would also generate Spi^- recombinants when the insertion is polar on the expression of the red and gam genes. The incorporation

of a chi mutation into the right arm of these proposed vectors would enhance the growth of these Spi$^-$ recombinants on the selective host, allowing better yields to be obtained. These XhoI vectors would provide another cloning system with a direct selection for in vitro recombinants.

Detection of chi-like sites in the genomes of a phage and its host

Two methods were used to test substituted or in vitro recombinant lambda DNA for chi-like sites. Both rely on the loss of the phage N-gene function, either by growth of phages carrying two nonsense mutations in the N-gene and the ninR5 deletion on a suppressor-free host, in which case only the amino-terminal fragments of the N protein are made and there is no detectable expression of N function, or by growth of ninR5 derivative phages on groN785, a host which is insensitive to the lambda N protein and blocks its anti-termination function. In both cases the phage late genes are expressed independently of N gene control due to the leakiness of t_{R1} and the deletion of t_{R2}, but leftward transcription of the N-operon itself is blocked by t_{L1} and hence the red and gam genes (necessary for efficient phage DNA replication and maturation) are not expressed.

The NNnin phages were propagated on C600(supE) or occasionally on Ymel(supF) as the permissive host. Reichardt (1975) has shown that the supF suppressed Nam7am53 product is less effective than the wild-type in stimulation of cI (lambda repressor) expression, and hence it may be that the red and gam products are also undersupplied. This could confer a slight advantage on phages

carrying a chi-site and thus constitute a residual selection for new spontaneous chi mutations during the growth of the phage stock on a supF host at any time during its history. The groN test is free from this worry as the test phages carry a wild-type N gene, but, since results obtained with the trp-transducing phages by the two methods agree, any remaining selection for new chi sites in the NNnin derivatives must be very weak.

An important limitation of the tests described is the effect of the genome size of the test phages on both their burst size and their plaque morphology, as illustrated by the case of λbio16A. This phage carries the naturally-occurring chi site in the E.coli bio operon, but due to its DNA excess (103.8% lambda length) its Spi⁻ derivatives give a poor burst and small plaques (Malone & Chattoraj, 1975). The phages λbio16ANNnin and λbio72NNnin tested here both have genome sizes of 97.5% wild type (Hradecna & Szybalski, 1969) and both carry the bio-chi site and yet show a two-fold difference in their burst sizes on the sup° host W3350 (Figure 4-1). They differ in the extent of their substitutions, bio16A removes only 1.5% lambda length, including the int gene, whereas bio72 removes 5.0%, including all the genes between att and red (Szybalski & Szybalski, 1974). The data of Malone & Chattoraj also shows a small (1.0 - 1.5 fold) enhancement due to the presence of the b522 deletion (of 6.9% - slightly longer than that of the bio72 substitution). The results of the groN-tests presented here also show an enhancement of the growth of the λ722 derivatives over those of λ540, at least when each lacks a chi-like site. The

vector λ722 differs from λ540 in only two particulars: it carries a deletion (of about 5% lambda-length) running from *att* towards *red*, and also an amber mutation in the *gam* gene. The latter is unlikely to affect growth on the *gro*N-host in which it is not expressed, and the former resembles the *b*522 deletion. It seems therefore that there may after all be a gene or site between *int* and *red*, the deletion of which provides a small (1 - 2 fold) increase in the burst sizes of Spi$^-$ phages as postulated by Zissler *et al.*, 1971 but that this is obscured by the more marked (3 fold) *chi* effect.

The size of the genome and the constitution of the N-operon of the phages tested are thus both important in the assignment of *chi*-like sites, but only in the case of λ*cys*B, where the 7 - 8% lambda length substitution of bacterial DNA brings the genome to the lower limits of viability, do these considerations prevent the confident assignment of the presence or absence of *chi*-like sites.

The detected sites are referred to as *chi*-like to show that the several possible origins of the observed phenotype have not been distinguished, e.g. the expression of a new *gam*-like activity or a *cro*$^-$ mutation. The acquisition of a *gam*-like protein would block the *rec*BC specified exonuclease V activity and allow leakage (in the absence of *red* function) into the rolling circle mode of phage DNA replication, giving a larger burst and a healthier plaque. In contrast, an additional *cro*$^-$ mutation, which has been shown to alleviate the effects of an NN$^-$ defect on N-operon expression (Court & Campbell, 1972), would allow normal phage replication

and maturation with consequent wild-type burst sizes and plaque morphology. The frequency of independently isolated chi-like sites argues against the first alternative and the mapping of several of the sites to within substituent DNA shows that the latter does not apply in these cases. The sites observed are thus probably true chi sites, but it remains to be shown that they exert their effect by a localised enhancement of rec-mediated recombination.

The groN785 strain was selected as a survivor after mutagenesis and challenge by high multiplicities of λc and λimm434c (Georgopoulos, 1971) and these phages show a very low eop on this host (10^{-11} and 5.10^{-8} respectively). Phages 434 and 21 also grow poorly on groN785 : eop's of 10^{-7} and 10^{-5} respectively, but as described in Chapter 4, λimm21 grows well (eop approaching 1.0).

Lambda and its imm434 derivative have the same N-gene, but differ in the interpenetrating operator and promoter sequences of their major leftward and rightward control complexes (Blattner & Dahlberg, 1972; Blattner et al., 1972) where the N protein acts (Franklin, 1975); so the small differences observed between them must mean that the lambda N protein retains slightly more activity at the imm434 promoters than those of lambda itself when it infects a groN host.

The imm21 derivative of lambda has both the N gene and the major control complexes ($o_L p_L$ and $p_R o_R$) of phage 21, and yet, whereas these are together ineffective in overriding the termination of transcription at its own (phage 21) terminators in a groN host, the

same N protein acting on groN host transcription machinery also at the promoters of phage 21 is able to efficiently overcome the lambda terminators in the hybrid. Thus, somewhat surprisingly, whilst both lambda and phage 21 are cut back by groN785 the hybrid λimm21 is not. Also, since the original imm21 phages, λ540trpA and λ540trpC show no difference in plaque morphology, the effect of chi-like sites on the growth of λimm21 phages must be small and the red and gam products cannot be limiting for the replication and maturation of these hybrids.

The analysis of λninR5 phages containing substitutions from Ø80 in the left arm show the presence of at least two chi-like sites in the Ø80 genome. This knowledge should be useful in the manipulation of λred⁻gam⁻ phages, which are difficult to propagate as they exhibit the partial Spi⁻ phenotype : giving very small plaques and low bursts even on permissive hosts; but with an $h^{Ø80}$ or $att^{Ø80}$ substitution, each bringing with it a chi-like site, these red⁻gam⁻ derivatives should now show the much more vigorous growth of the full Spi⁻ phenotype (Henderson & Weil, 1974; Malone & Chattoraj, 1975).

Results of tests for chi-like sites in the E.coli trp-operon DNA reveal the presence of at least two such sites, and deletion mapping places them both within the region ~~augmented~~ covered by trpD1, one on either site of the HindIII target in the trpD gene. The orientation of the substituent DNA carrying the chi-like site(s) does not appear to affect the phenotype conferred, though previous

work has shown that in the case of mutationally acquired chi sites in the lambda genome the stimulation of recombination is strongly polarised (Stahl & Stahl, 1975). Further deletion mapping of the chi-like sites in the trpE$^+$ and trpC$^+$ *in vitro* recombinant transducing phages will be necessary to localise them more accurately before nucleic acid sequencing studies become attractive.

Similar scans of the E.coli DNA carried by *in vitro* recombinant specific transducing phages have revealed seven chi-like sites in a survey of an estimated 2.5% of the E.coli chromosome, and the extrapolation of these results lead to an expectation of about 250 chi sites in the whole bacterial genome. It is interesting to note that of the two cases where any details are known, those of the trp and bio regions of the E.coli chromosome, the sites lie within the structural genes (of trpE, trpD and bioB, respectively). One could speculate that enhanced recombination in the region of important structural genes may have some evolutionary significance for the organism, perhaps by directing recombination among functional loci and thus allowing more rapid assimilation of mutations among a population.

Cases of 'marker effects' where particular mutations modify the normal frequency of recombination in their environs are well documented (Yanofsky *et al.*, 1964; Norkin, 1970) and it would be interesting to know if any of these effects are due to the creation or destruction of chi sites by the mutation. Should such cases come to light, as is already known for the lambda χ_c mutants in the cII gene (McMilin *et al.*, 1974), nucleic acid sequencing

studies of the wild type and the mutant would allow identification of the elements of sequence which specify a chi site.

Consideration of data available at present suggest that the nucleic acid sequence specifying a chi-site is short : Since there are four sequences in the lambda genome which can become chi-sites by a single base change (McMilin et al., 1974; Henderson & Weil, 1975), such potential chi sites occur on average about once per 12 Kb. The frequency of random occurrence of a particular heptanucleotide sequence is once every 12.4 Kb (4^7 base pairs), so it seems plausible that a chi site could be specified by a sequence of eight bases, though these may be interspersed with random elements.

Investigation of lambda NN$^-$ plasmid vectors

The design and construction of lambda plasmid vectors offers the prospect of molecular cloning vehicles with many of the advantages of other plasmid vectors, in a genome whose regulation is well understood and providing a simple direct method for selection of plaque-forming transducing phage derivatives by transfer of the plasmid DNA to a suppressing host. The propagation of these lambda plasmids is regulated by the cro product (See Introduction).

Superinfecting λc is sensitive to repression by the high levels of cro product in a host bearing a lambda plasmid, and so anti-immunity is displayed, but λvir escapes this repression by virtue of its mutant major control sites (Jacob & Wollman, 1954; Ptashne & Hopkins, 1968; Horiuchi et al., 1969; Koga et al., 1970)

which are less sensitive to cro-repression as well as to the lambda repressor product of the cI gene. Thus, as seen in strains harbouring the proposed lambda plasmid vectors, λc cannot form a plaque on a (λNN⁻) anti-immune host, whereas λvir is able to do so. This, incidentally, provides evidence for the interpenetration of the major operators and sites of cro action.

Establishment of lambda plasmids after infection with the λNN⁻ vector phages is two to three-fold less efficient than plaque formation, but this has also been observed with λgalNN plasmids (Signer, 1969).

Suppressor-free strains with a high competence for DNA uptake, and carrying small internal deletions in the trp and lac genes, were constructed as hosts suitable for transformation by the proposed lambda plasmid vectors. These strains, A125 and A129 and their supF (trp⁺) derivative A126, show very efficient DNA uptake by present standards : yielding 2.5×10^6 pfu/ug intact lambda DNA added, and only slightly fewer transformants per ug of λNN⁻ DNA in the respective selective systems. This efficiency corresponds to one infectious centre, or transformed clone per 10^4 lambda DNA molecules added : an overall efficiency of only 0.01%. Thus, there is a substantial margin for improvement, both by the use of better transformation/transfection techniques and by the use of more highly competent host strains which would allow more efficient recovery of in vitro recombinant DNA.

Two systems for the selective propagation of transformant, lambda plasmid carrying clones were investigated. The first relies on the ability of the host, when bearing a λlacNN:64 plasmid, to grow on lactose as the sole carbon source, whereas the uninfected cells alone cannot. This host defect is due to the laczM15 deletion, which can be complemented by the lacz$^+$ plasmid or by the laczα^+ *in vitro* recombinant lambda plasmids (the former by allelic complementation and the latter by intra-allelic, or alpha-omega complementation). This selection proved to be too weak to be useful with the host strains A125 and A129, but parallel experiments with another laczM15 strain : XA21, showed a much stronger growth response under the selective conditions, even by the weaker intra-allelic complementation of the host laczM15 product by the lacz-alpha fragment specified by the λ806NN plasmid. XA21, however, is a poor transformation host, but this latter result shows that the system may yet be useful should a lacz-omega donor strain with a high competence for DNA uptake be found. *In vitro* recombinant plasmids would then be identified among the Lac$^+$ clones as smaller colonies, due to the weaker alpha-omega complementation conferred by plasmids with foreign DNA inserted into the lacz gene, as compared with the strong Lac$^+$ phenotype characteristic of the intact λlacNN:64 plasmid vector.

The second selective system was ampicillin resistance conferred by a plasmid carrying the transposon TnA, which specifies the TEM β-lactamase, and this was shown to provide a strong selection for plasmid-bearing clones. Attempts to ligate foreign DNA insertions

into the single EcoRI site of the λampNN:63 plasmid, though promising, have not yet been conclusively demonstrated. $Trp^{+}Amp^{R}$ clones have been selected after ligation in the presence of a digest of a trp-transducing phage, but these proved to be unstable. However, these in vitro recombinant lambda plasmids were recovered by transformation of a $trpR^{-}$ strain, and this combination of transcription of the trp-operon from a high copy-number plasmid in a genetically derepressed background has previously been reported to be unstable (Herschfield et al., 1974). Similarly, these workers showed that stable incorporation of the trp genes from Ø80pt190 was only achieved when the phage repressor gene was also cloned in the same plasmid (even though they are carried by discrete EcoRI fragments) allowing repression of transcription of the operon from the phage promoter p_L. Crabeel et al. (1977) have shown a similar instability of arg-transforming plasmids in $argR^{-}$ hosts. Thus it seems not unreasonable to infer that high level transcription of these multi-copy plasmids deprives the host of the capacity to supply its own essential products and results in loss of viability, or loss of the plasmids.

DNA insertions into the λampNN:63 vector at srIλ2 would not be transcribed from a phage promoter in the plasmid, whereas insertions into the EcoRI site in the lacz gene in λlacNN:64 could be expressed from the lac operon promoter. This transcription could be easily manipulated by well-tried techniques (See for example Polisky et al., 1976 : 'A plasmid cloning vehicle allowing regulated expression of eukaryotic DNA in bacteria.'). This would allow the

selection of recombinants containing genes which are separated from their own promoter, or the fused lac promoter might be used to boost the synthesis of gene products which are normally only weakly expressed.

A λNN⁻ vector carrying both TnA and plac5, and with the single srIlac target, would combine the advantages of a strong selective system for transformed clones and the expression of cloned EcoRI fragments from the lac promoter, and if transformants were selected on a lacz⁻ (total z deletion) host on lactose-McConkey agar containing ampicillin, recombinant clones would be identifiable as white (Lac⁻) colonies among the red (Lac⁺) vector-containing clones.

The recombinant plasmids could be transferred to other hosts for complementation tests, either by recovery of CCC DNA and retransformation of the appropriate sup° host (selecting Amp^R clones), or by P1 mediated transduction of the plasmid into the sup° host, as demonstrated by Ramakrishnan & Adelberg, (1965), and Novick, (1969).

In summary, then, lambda NN⁻ plasmid vectors offer several novel advantages as molecular cloning vehicles, but an ideal vector of this type is not yet available.

APPENDIX I

Changes in the linear structure of a DNA molecule, their assignment and their effects on restriction patterns of these molecules

The following types of changes in the linear structure of a double-stranded DNA molecule will be considered:

(a) Deletion or accretion

(b) Substitution

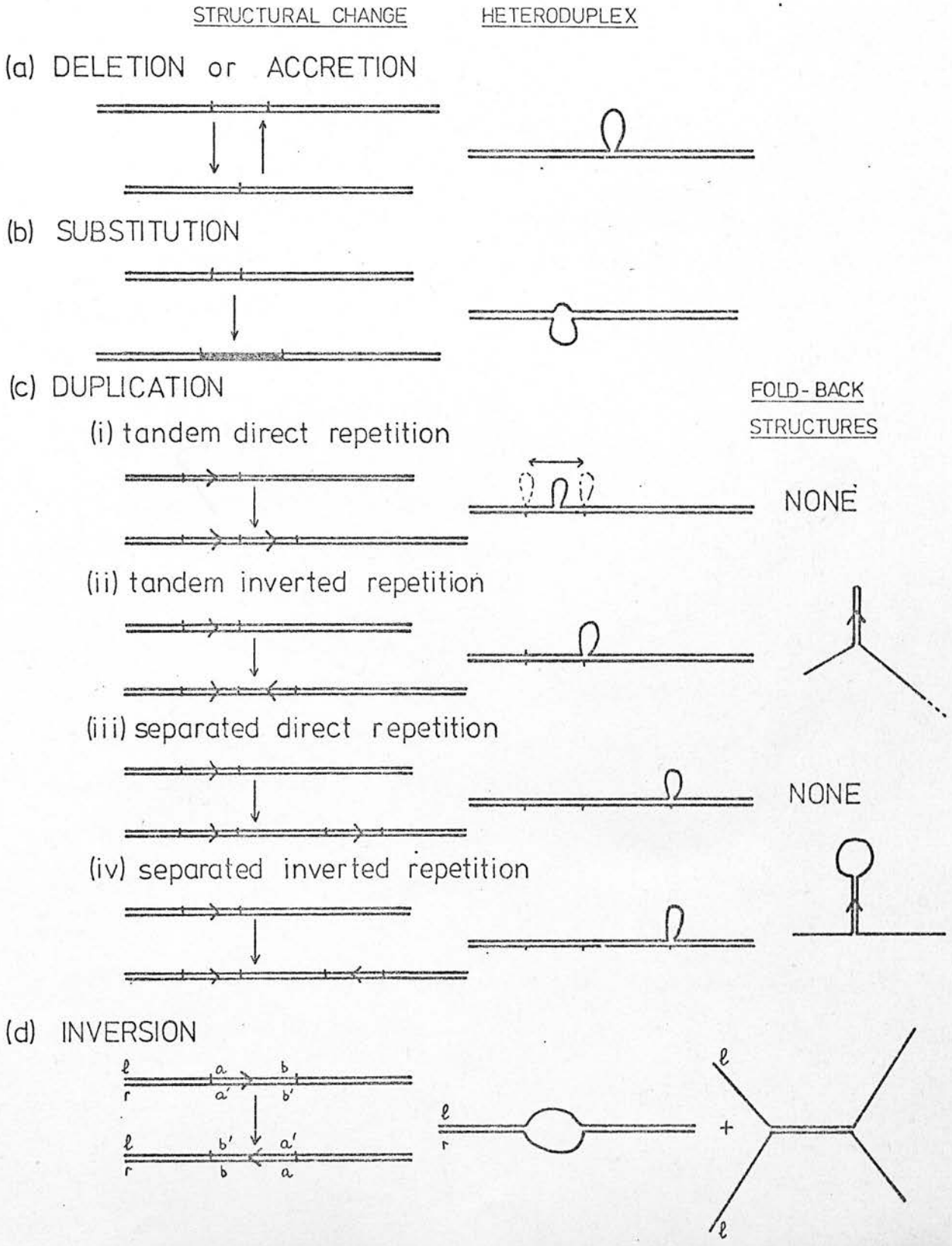
(c) Duplication

(d) Inversion

The character of a particular change is most easily diagnosed by examination of an heteroduplex formed between complementary strands of the related molecules. The formamide spreading technique is designed to show both double and single stranded regions in an extended, measurable form by providing sufficiently denaturing conditions to overcome random interactions in single stranded regions without interfering with the pairing of fully complementary double stranded regions. The form of the change relating the two molecules can then be deduced by inspection.

Figure A-1 is a schematic representation of the different types of heteroduplex which may be obtained from a pair of DNA molecules related by a single structural change. A simple deletion removes a continuous stretch of the linear molecule so that when an heteroduplex is formed from one strand of the original molecule and the complementary strand of its deletion derivative a single stranded loop is seen where the parental molecule possesses a stretch of sequence to which no corresponding complement is present in the derivative, as shown in Figure A-1(a). It is clear

FIGURE A-1 Effects of structural changes on reannealing patterns of DNA molecules



that a simple accretion, or addition of a stretch of sequence interposed at some position along a DNA molecule represents the exact inverse of a deletion event, and the form of the heteroduplex of the two related molecules of these two classes will be indistinguishable. To assign such a structure to one or the other type of change requires a single additional piece of information: namely the knowledge of which molecule (the original or its derivative) is the longer.

A substitution, case (b) in Figure A-1, arises where a continuous stretch of sequence within a molecule is deleted and a different sequence inserted at the same position, i.e. one sequence is replaced by another and their relative lengths determine the net size change. The heteroduplex formed from complementary strands of molecules related in this fashion shows a 'bubble' where two single stranded arms of non-homologous sequence link the two double stranded regions.

A duplication is a special case of the accretion class in which the added linear sequence is a second copy of one already present in the original molecule. Duplications fall into several discrete categories, each with different heteroduplex characteristics. Figure A-1(c) shows the orientations and relative positions of the duplicated sequence which distinguish the four types. When the duplicated segment is contiguous with its copy the arrangement is referred to as 'tandem', whereas the alternative is a 'separated' configuration. Further, taking into account the constraints imposed

by the 5'-3' polarity of the DNA strands, the duplicated segment may be oriented in either of two senses with respect to the pre-existing copy, thus giving the four possible arrangements shown (i-iv). Conventional heteroduplex molecules incorporating one strand of the original molecule and the complementary strand from its duplication derivative always show a single stranded loop due to one of the two copies on the latter strand. The diagnostic feature of molecules containing duplications is the intramolecular reannealing of their isolated single strands to form 'fold-back' structures, the form of which in itself identifies the duplications as being tandem or separated, direct or inverted: Figure A-1(c). Confirmatory evidence for each assignment comes from their heteroduplex forms: a direct tandem repetition yielding a loop whose base on the double stranded region is continuously variable (for different heteroduplex molecules) between the boundaries of the repeated segment (i), whereas an inverted tandem repetition, and either form of repetition at a location distant from the original copy shows a unique accretion loop (ii-iv).

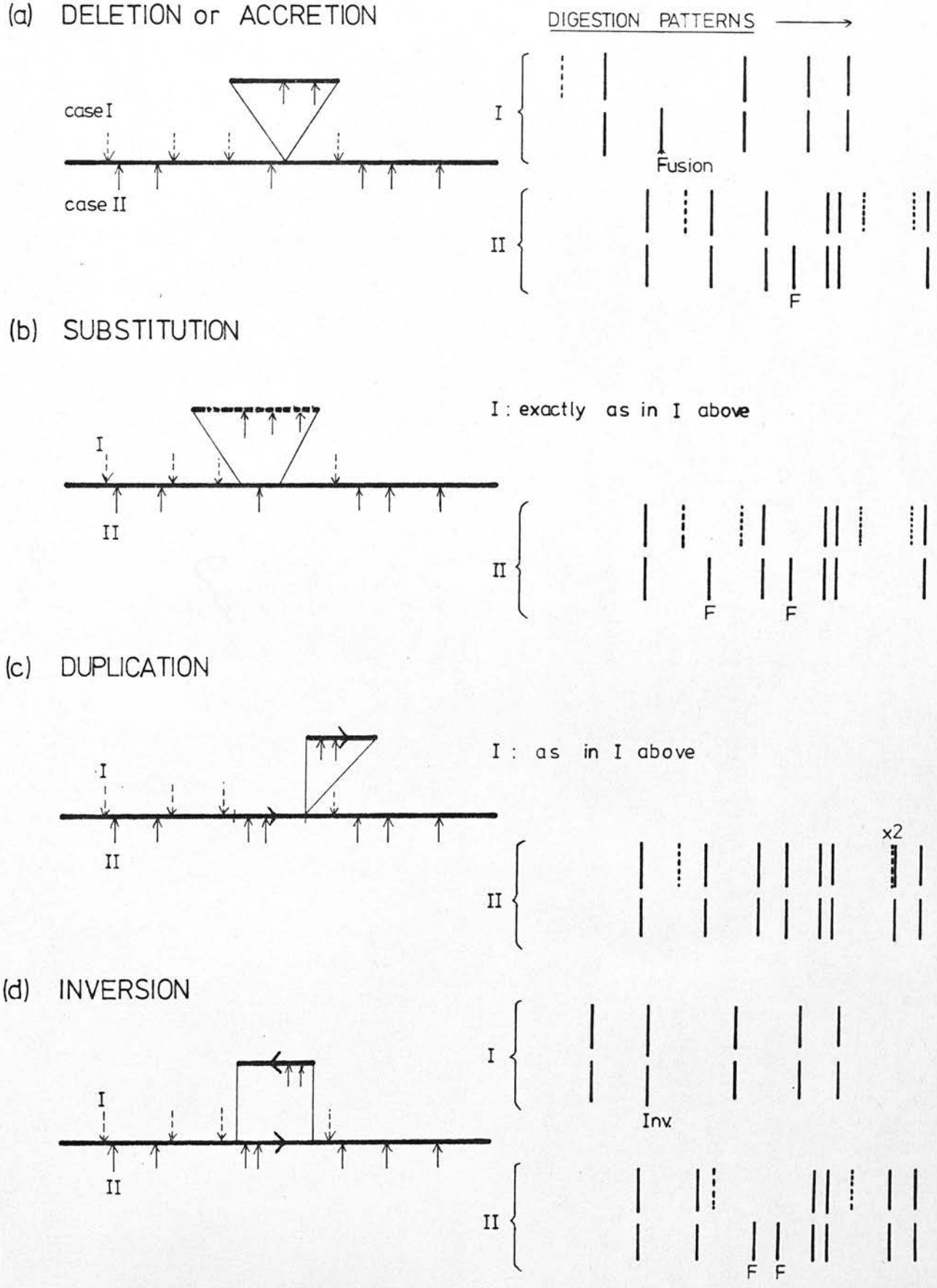
An inversion occurs where a segment $\overset{a}{a'}\!\!=\!\!=\!\!=\!\!=\overset{b}{b'}$ of the molecule is excised and rejoined in the orientation $\overset{b'}{b}\!\!=\!\!=\!\!=\!\!=\overset{a'}{a}$, maintaining the 5'-3' integrity of each of the strands of the intact molecule. Isolated single strands of these molecules show no intramolecular reannealing, but conventional 'l' or 'r' strands of the parental molecule and its inversion derivative show complementarity along the length of the inversion: Figure A-1(d).

The limits of detection of these changes in the linear structure of a DNA molecule by the heteroduplex method depend on

the ability to resolve small regions of non-homology within an otherwise fully complementary double stranded molecule, and also on the efficiency of reannealing of short stretches of complementary sequence within otherwise non-homologous regions. A deletion loop of between 100 and 140 nucleotides in length (containing one of the tyrosine-tRNA copies) was easily detected and clearly resolved in the Ø80suIII^+suIII^-/Ø80suIII^+ heteroduplex (Miller et al. 1971). The λ^+/Ø80^+ heteroduplex shows a limited homology at the extreme right tip in which the short double stranded region is not resolved; comparison of the frequency of formation of this short double stranded segment with that of circularisation of λ DNA molecules by reannealing of the 12 base-pair cohesive end sequences suggests that the right tip homology may be as little as 12 nucleotides in length, (Fiandt et al. 1971). Thus deletions, accretions, substitutions, duplications or inversions of sequences longer than 100 nucleotide pairs are readily detected by this method, and interstitial regions of homology of much shorter sequences (probably less than 50 base pairs) may be discerned.

The effects of the structural changes discussed above on restriction fragment patterns obtained with the 'Type II' (Arber & Linn, 1969) sequence-specific restriction endonucleases will now be considered. Restriction digests of DNA molecules related by a simple deletion (or an accretion) are compared in Figure A-2(a), (i) and (ii) representing two extreme cases. The following discussion

FIGURE A-2 Effects of structural changes on restriction patterns



will be of a deletion event; in the case of an accretion the parental and derivative molecules are interchanged. A deletion removes one or more fragments and a single fusion-fragment is formed from the two sections of the terminal fragment, only part of which are removed by the deletion. When sites for the restriction enzyme lie under the deletion, as in case (ii), all fragments between these sites are lost from the digest of the derivative. It will be clear from Figure 2A that a substitution (dotted) may lead to the appearance of more than one new fragment in the digest of the substituted molecule, and two fusion fragments may be formed, (b).

A duplication (c), will resemble an accretion when examined by restriction analysis, although several fragments present in the original digest may be represented twice in that of the duplication derivative.

Finally, an inversion (d), may not be detected at all in the restriction digest (I) but will be most clearly seen where there are asymmetrically distributed sites in the inverted segment (II), producing two new fusion fragments.

In summary, then, the nature of a structural difference between two DNA molecules can most easily be assessed by heteroduplex analysis, giving its size and position. In the light of this information, restriction fragment patterns are much more easily interpreted. This approach is restrained only by the lower limits of resolution of heteroduplex homologies and non-homologies, and by the difficulty of detection of small, or resolution of identically sized, restriction fragments.

Appendix II

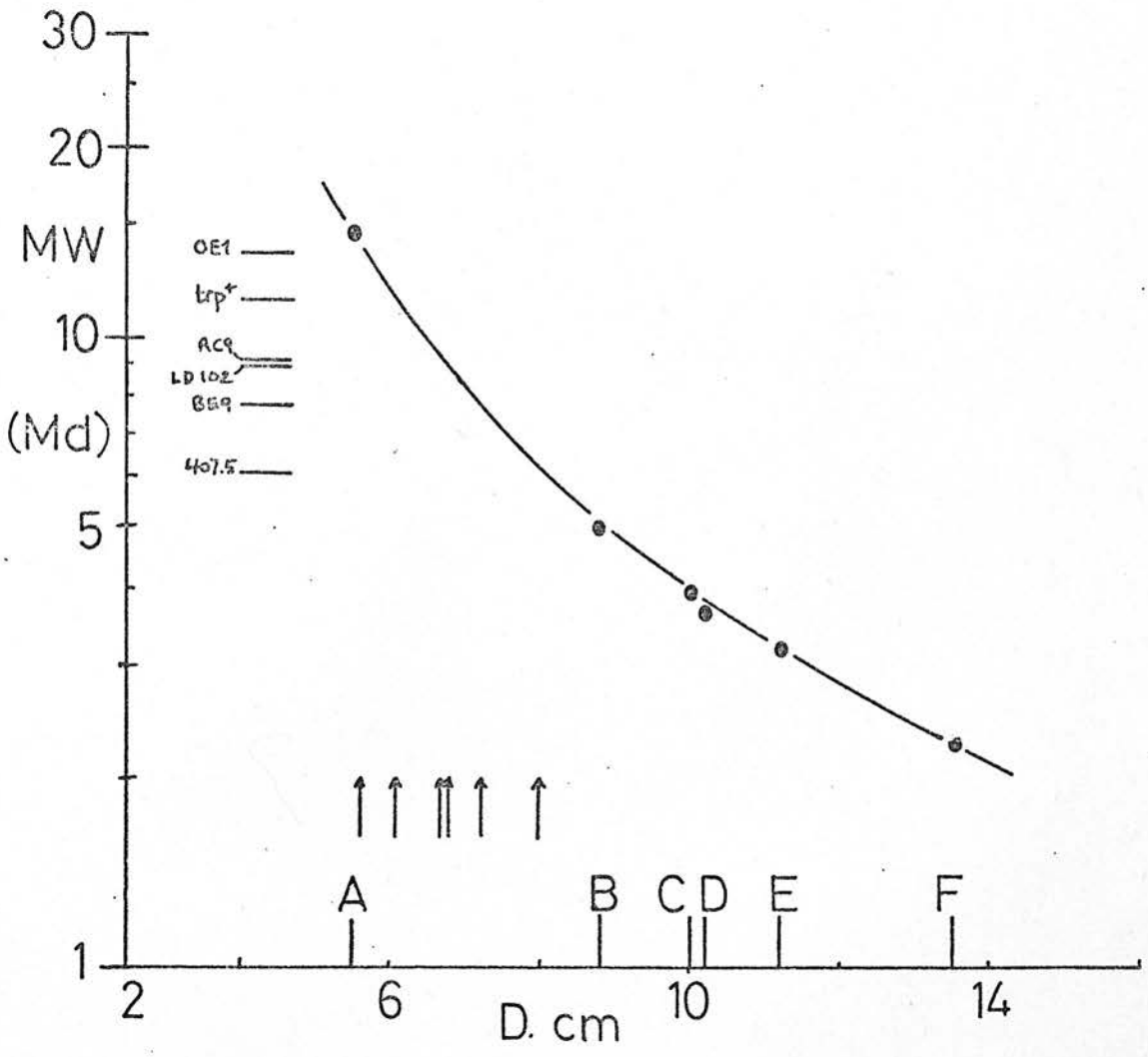
Determination of molecular weights of DNA fragments

Two gel electrophoresis systems were used during the course of the work described: Firstly, and more routinely, 1% agarose gels run in the continuous E buffer system of Hayward & Smith (1972), for the determination of molecular weights from a lower limit of approximately 0.3 Md, or about 450 base pairs, upto the full lambda size of 33 Md : 49 Kb. This system is very insensitive to small molecular weight differences between fragments of more than about 5 Md, but is quite sensitive in the lower molecular weight range, where the mobility of each fragment is a linear function of the logarithm of its molecular weight, as will be demonstrated.

Secondly, for fragments of less than about one thousand base pairs in length (0.66 Md), to an estimated lower limit of 150 base pairs or 0.1 Md, a linear gradient polyacrylamide gel, again in E buffer, was used (Jeppensen, 1974). This system is reported to give an overall linear response of mobility vs molecular weight, at least down to 300 base pairs (0.2 Md), but unfortunately is markedly affected by the base composition of the migrating fragments (Zeiger et al., 1972 and Allet et al., 1973) and is less reliable than the agarose gel system for this reason.

Figure A-3 is a semi-logarithmic mobility (distance migrated) vs molecular weight from data shown in Plate 6. Only tracks 1 - 8 were measured, as edge-effects are apparent in the last two tracks causing slight bowing of the bands. The known sizes of lambda fragments generated by EcoRI digestion (Thomas & Davis, 1975 as modified by Philippsen & Davis, 1977) were used to generate the

FIGURE A-3 Mobility curve for Plate 6



• λ+: EcoRI

				Migration
A	14.4 Md	21.8 Kb	44.5 %λ+	5.5 cm
B	4.95	7.52	15.4	8.8
C	3.92	5.93	12.1	10.0
D	3.65	5.54	11.3	10.2
E	3.17	4.80	9.8	11.2
F	2.25	3.41	6.9	13.5

calibration curve, and the molecular weights of the EcoRI : trp DNA fragments of the λtrp51 series of phages determined as shown.

Figure A-4 is a similar plot from the data of Plate 7. The molecular weights of the fragments in a HindIII digest of lambda (Philippsen & Davis, 1977) and those of several HpaII fragments of lambda (Allet, 1973) were used to plot the curve. The molecules of the characterised HpaII fragments (shown in Plate 20) were determined by comparison with co-migrating HindII & III fragments of lambda present as markers in both gels.

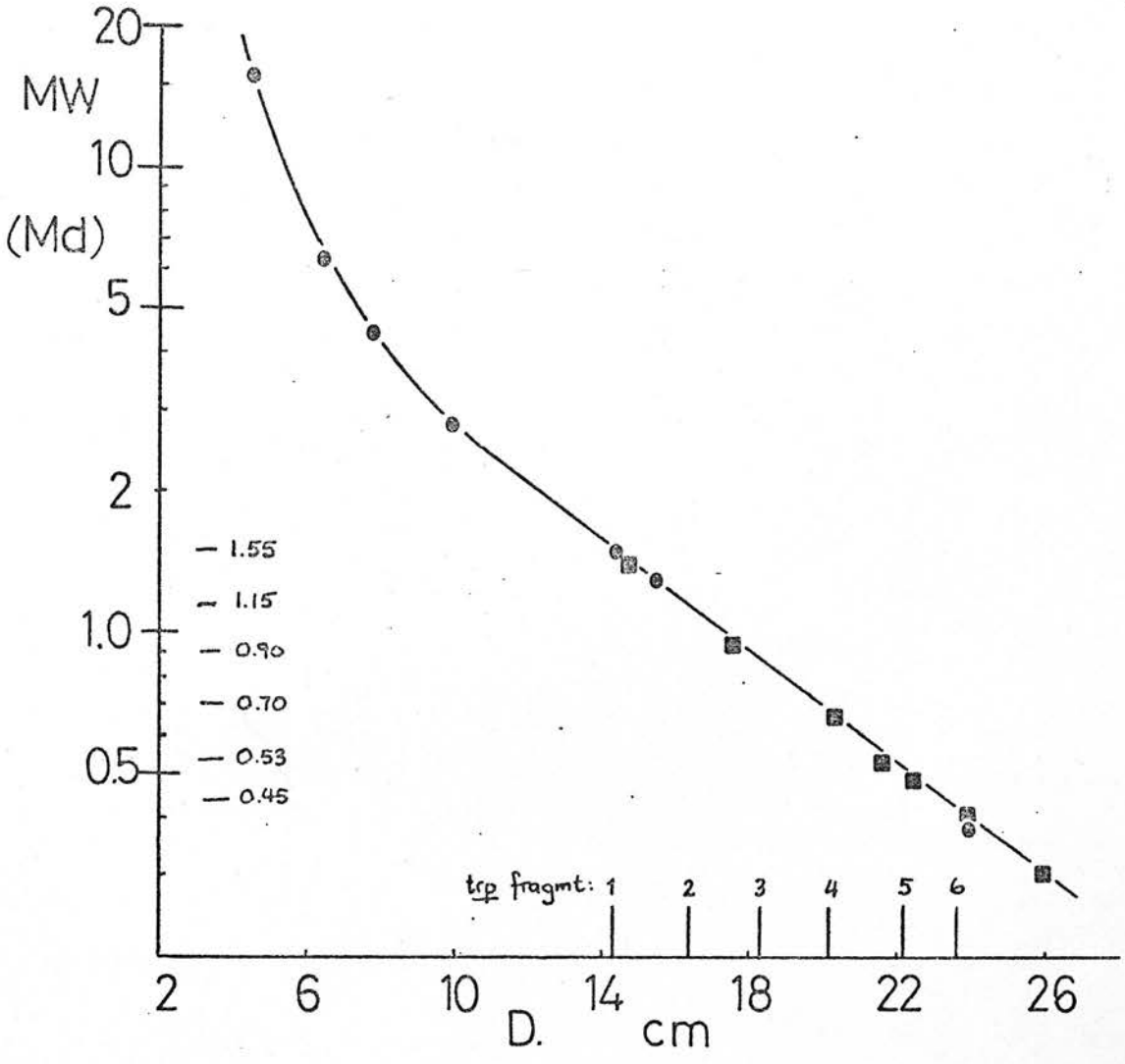
Figure A-5 shows a linear plot of the mobility of the known HpaII fragments of lambda DNA on the polyacrylamide gel of Plate 8. Because the markers used are much larger than the smallest trp fragment seen, its molecular weight remains undetermined but is probably less than 250 base pairs.

Mobility curves were drawn from the data of Plates 11 and 12, using HindIII fragments of lambda DNA for calibration points in each, just as in Figure A-4, the curves (not shown) being of the same form and accuracy, yielded the following data:

HindIII : trp DNA fragments (from Plate 11)

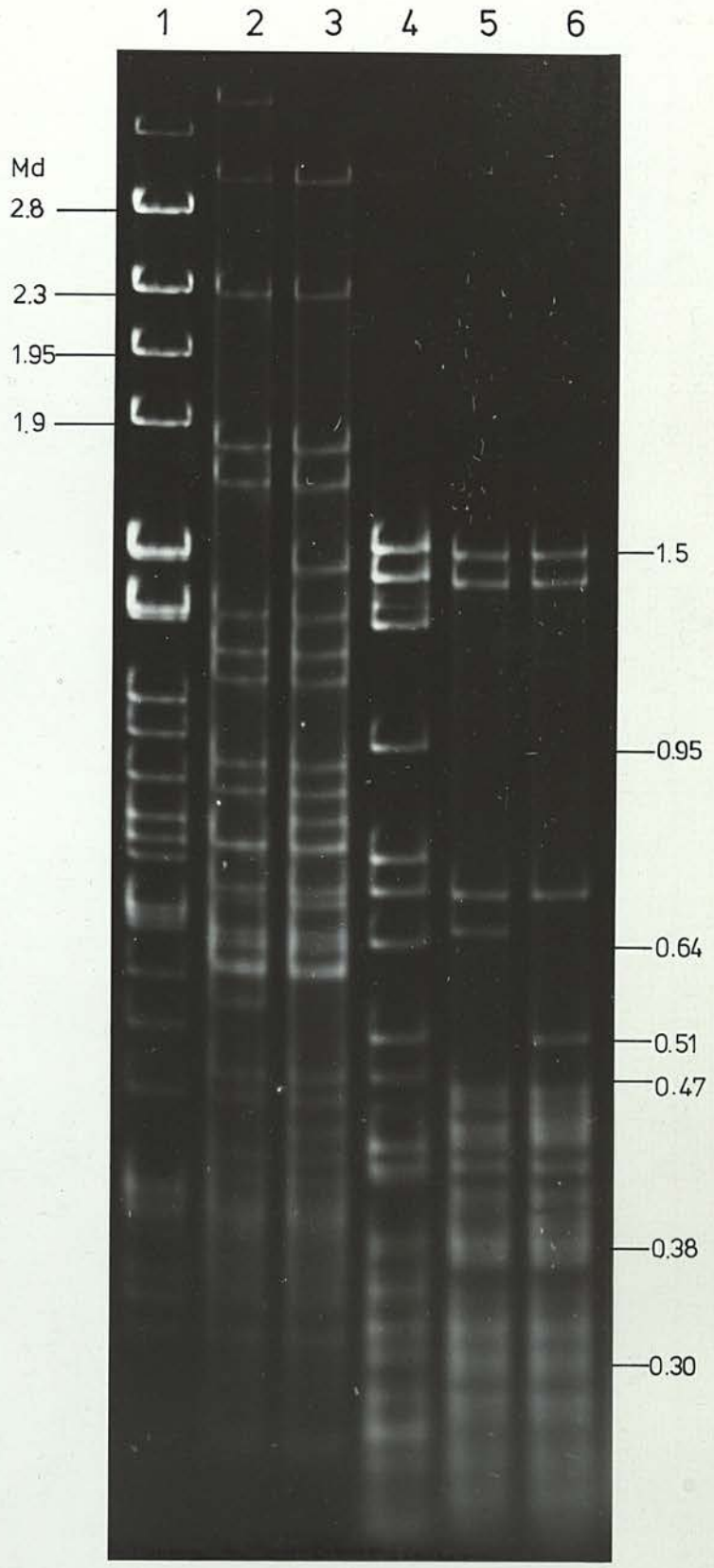
	Molecular weight (Md)	Size (Kb)
1	6.5 ± 0.5	9.8 ± 0.75
2	4.8 ± 0.4	7.2 ± 0.6
3	1.9 ± 0.1	2.9 ± 0.15

FIGURE A-4 Mobility curve for Plate 7



■ λ+: HpaII ● λ+: Hind III

				Migration
A	15.7 Md	23.9 Kb	48.3 %λ+	4.5 cm
B	6.25	9.46	19.3	6.4
C	4.45	6.75	12.7	7.7
D	2.82	4.26	8.7	9.9
E	1.49	2.26	4.6	14.3
F	1.31	1.98	4.05	15.4
G	0.38	0.58	1.18	23.9



HindII+III digests:

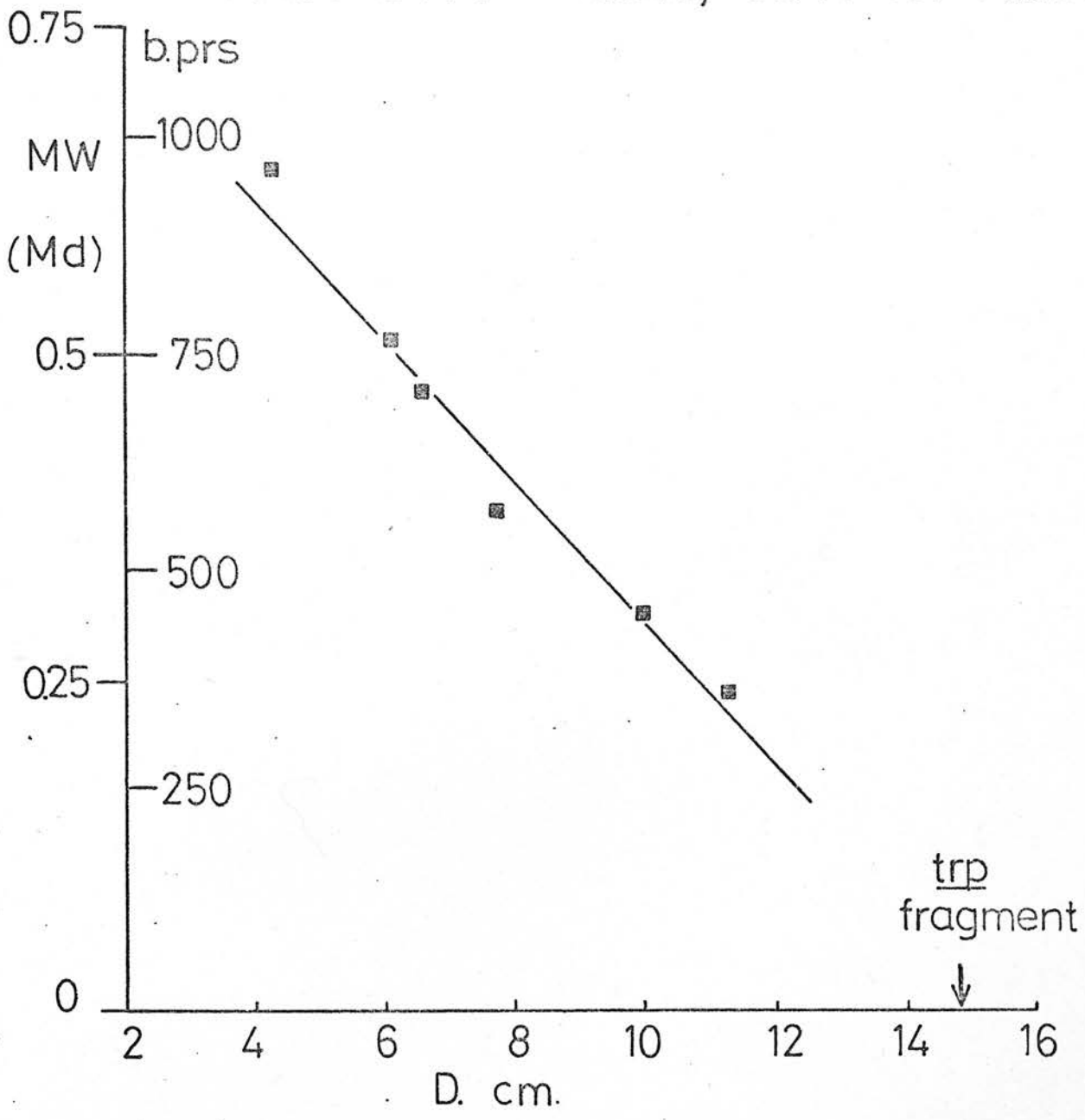
1. λ⁺
2. λ<u>trp</u>51∇LD102
3. λ<u>trp</u>⁺51

<u>Hpa</u>II digests:

4. λ⁺
5. λ<u>trp</u>51∇LD102
6. λ<u>trp</u>⁺51

Plate 20

FIGURE A-5 Mobility curve for Plate 8



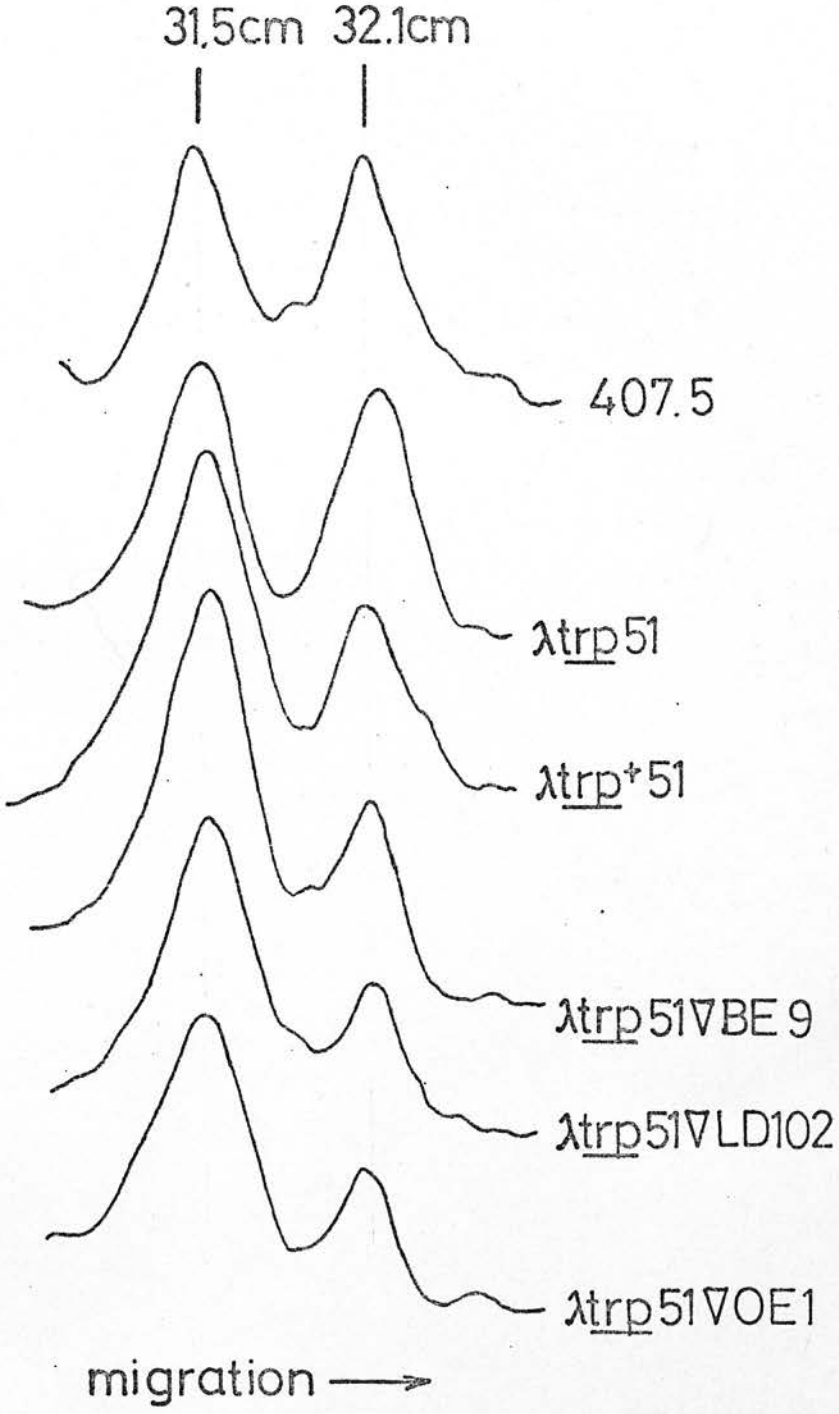
■ λ⁺: HpaII frags

	Molr. wt (Md)	Migrⁿ (cm).
1	0.64	4.5
2	0.51	6.1
3	0.47	6.5
4	0.38	7.7
5	0.30	10.0
6	0.24	11.3

Plate 12	HpaI : trp DNA fragments:		
digest:	migration (cm)	Molecular weight (Md)	Size (Kb)
λ trp51	15.5	3.95 ± 0.3	5.92 ± 0.45
λ trp^{+}51	20.0	2.15 ± 0.1	3.22 ± 0.15
λ trp51 ∇ LD102	22.6	1.65 ± 0.07	2.48 ± 0.1
λ trp51 ∇ BE9	31.1	0.68 ± 0.03	1.02 ± 0.05

Figure A-6 shows the densitometry traces of the doublet band at 31.5 cm on the negative of Plate 14. Despite different DNA loadings it is clear that the band at 31.5 cm is present in two copies in the AvaI digests of all the λtrp51 derivatives except λtrp51 itself and its hybrid parent phage 407.5.

FIGURE A-6 Densitometry traces of AvaI doublets



REFERENCES

ALLET, B. (1973) Biochemistry 12 : 3972-3977

ALLET, B. & BUKHARI, A.I. (1975) J.Mol.Biol. 92 : 529-540

ALLET, B., JEPPENSEN, P.G.N., KATAGIRI, K.J. & DELIUS, H. (1973) Nature (London) 241 : 120-123

ALLET, B., ROBERTS, R.J., GESTELAND, R.F. & SOLEM, R. (1974) Nature 249 : 217-221

ALLET, B. & SOLEM, R. (1974) J.Mol.Biol. 85 : 475-484

APPLEYARD, R.K. (1954) Genetics 39 : 440-452

APPLEYARD, R.K., McGREGOR, J.F. & BAIRD, K.M. (1956) Virology 2 : 565-574

ARBER, W. & LINN, S. (1969) Ann.Rev.Biochem. 38 : 467-500

BACHMANN, B.J. (1972) Bact.Rev. 36 : 525-557

BACHMANN, B.J., BROOKS LOW, K. & TAYLOR, A.L. (1976) Bact.Rev. 40 : 116-167

BARBOUR, S.D. & CLARK, A.J. (1970) Proc.nat.Acad.Sci. (Wash.) 65 : 955-961

BELLET, A.J.D., BUSSE, H.G. & BALDWIN, R.L. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY 501-513

BENNET, G.N., SCHWEINGRUBER, M.E., SQUIRES, C. & YANOFSKY, C. (1976) Proc.nat.Acad.Sci. (Wash.) 73 : 2351-2355

BENZER, S. (1955) Proc.nat.Acad.Sci. (Wash.) 41 : 344

BERG, D.E. (1974) Virology 62 : 224-233

BERTANI, L.E. & BERTANI, G. (1970) J.Gen.Virol. 6 : 201-212

BERTRAND, K., KORN, L., LEE, F., PLATT, T., SQUIRES, C.L., SQUIRES, C. & YANOFSKY, C. (1975) Science 189 : 22-26

BERTRAND, K., SQUIRES, C. & YANOFSKY, C. (1976a) J.Mol.Biol. 103 : 319-338

BERTRAND, K. & YANOFSKY, C. (1976b) J.Mol.Biol. 103 : 339-350

BLATTNER, F.R. & DAHLBERG, J.E. (1972) Nature New Biol. 237 : 227-232

BLATTNER, F.R., DAHLBERG, J.E., BOETTIGER, J.K., FIANDT, M. & SZYBALSKI, W. (1972) Nature New Biol. 237 : 232-236

BLATTNER, F., FIANDT, M., HASS, K.K., TWOSE, P.A. & SZYBALSKI, W. (1974) Virology 62 : 458-471

BLATTNER, F., WILLIAMS, W.G., BLECHL, A.E., DENNISTON-THOMPSON, K., FABER, H.E., FURLONG, L.A., GRUNWALD, D.J., KIEFER, D.O., MOORE, D.D., SCHUMM, J.W., SHELDON, E.L., & SMITHIES, O. (1977) Science 196 : 161-169

BORCK, K., BEGGS, J.D., BRAMMAR, W.J., HOPKINS, A.S. & MURRAY, N.E. (1976) Molec.gen.Genet. 146 : 199-207

BOYER, H.W. (1971) Ann.Rev.Microbiol. 25 : 153-176

BRAMMAR, W.J., MURRAY, N.E. & WINTON, S. (1974) J.Mol.Biol. 90 : 633-647

BRONSON, M.L., SQUIRES, C. & YANOFSKY, C. (1973) Proc.nat.Acad. Sci. (Wash.) 70 : 2335-2339

BUCHWALD, M. & SIMINOVITCH, L. (1969) Virology 38 : 1-7

CAMPBELL, A. (1957) Virology 4 : 366

CAMPBELL, A. (1959) Adv. in Genetics 11 : 101

CAMPBELL, A. (1961) Virology 14 : 22-32

CAMERON, J.R., PANASENKO, S.M., LEHMAN, I.R. & DAVIS, R.W. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 3416-3420

CARTER, D.M. & RADDING, C.M. (1971 a,b) J.Biol.Chem. 246 : 2502-2512 and 2513-2518

CARTER, D.M. & SMITH, M.G. (1970) J.Mol.Biol. 50 : 713-718

CASSUTO, E. & RADDING, C.M. (1971) Nature New Biology 229 : 13

CATCHESIDE, D.G. & ANGEL, T. (1974) Australian J.Biol.Sci. 27 : 219-229

CHOW, L., DAVIDSON, N. & BERG, D.E. (1974) J.Mol.Biol. 86 : 69-89

CLARK, A.J. (1967) J.Cell.Phys. suppl. to vol. 70 No.2 (Part II) : 165-180

CLARK, L. & CARBON, J. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 4361-4365

COLLINS, C.J., JACKSON, D.A. & de VRIES, F.A.J. (1976) Proc.nat.Acad.Sci. (Wash.) 73 : 3838-3842

COUKELL, M.G. & YANOFSKY, C. (1971) J.Bact. 105 : 864-872

COURT, D. & CAMPBELL, A. (1972) J.Virol. 9 : 938-945

COURT, D. & SATO, K. (1969) Virology 39 : 348-352

CRABEEL, M., CHARLIER, D. & GLANSDORFF, N. (1977) Molec.gen. Genet. 151 : 161-168

CRAWFORD, I.P. & JOHNSON, L.M. (1964) Genetics 49 : 267-278

DAMBLY, C., COUTURIER, M. & THOMAS, R. (1968) J.Mol.Biol. 32 : 67-81

DAVIDSON, N. & SZYBALSKI, W. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY : 45-82

DICKSON, R.C., ABELSON, J., BARNES, W.M. & REZNIKOFF, W.S. (1975) Science 187 : 27-35

DOVE, W.F. & WEIGLE, J.J. (1965) J.Mol.Biol. 12 : 620

DOVE, W.F., INOKUCHI, H. & STEVENS, W.F. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY

DRAPEAU, G.R., BRAMMAR, W.J. & YANOFSKY, C. (1968) J.Mol.Biol. 35 : 357-367

DEEB, S.S., OKAMOTO, K. & HALL, B.D. (1967) Virology 31 : 289

ECHOLS, H. (1972) Ann.Rev.Genet. 6 : 157-190

ENQUIST, L.W. & SKALKA, A. (1973) J.Mol.Biol. 75 : 185-212

FEISS, M.,ADHYA, S. & COURT, D. (1972) Genetics 71 : 189-206

FIANDT, M., HRADECNA, Z., LOZERON, H.A. & SZYBALSKI, W. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY : 329-354

FRANKLIN, N.C. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY : 621-638

FRANKLIN, N.C. (1975) J.Mol.Biol. 89 : 33-48

FRANKLIN, N.C., & DOVE, W. (1969) Genet.Res. 14 : 151-157

FREIFELDER, D., CHUD, L. & LEVINE, E.E. (1974) J.Mol.Biol. 83 : 503-509

GEORGOPOULOS, C.P. (1971) In 'The Bacteriophage Lambda' 639-645 Ed. A.D. HERSHEY Cold Spring Harbor Lab.

GEORGOPOULOS, C.P. & HERSKOWITZ, I. (1971) ibid. 553-564

GILBERT, W. & DRESSLER, D. (1968) Cold Spring Harbor Symp.Quant. Biol. 33 : 473-484

GILBERT, W. & MAXAM, A. (1973) Proc.nat.Acad.Sci. (Wash.) 70 : 3581-3584

GILBERT, W. & MAXAM, A. (1977) Proc.nat.Acad.Sci. (Wash.) 74 : 560-564

GOLDBERG, A.R. & HOWE, M. (1969) Virology 38 : 200-202

GOLDSCHMIDT, E.P., CATER, M.S., MATHEY, T.S., BUTLER, M.A. & GREENE, A. (1970) Genetics 66 : 219-229

GOTTESMAN, M.M., GOTTESMAN, M.E., GOTTESMAN, S. & GELLERT, M. (1974) J.Mol.Biol. 47 : 565-574

GREENSTEIN, M. & SKALKA, A. (1975) J.Mol.Biol. 97 : 543-559

HAGGERTY, D. & SCHLIEF, R. (1976) Virology 18 : 659-663

HARDMAN, J.K., BERGER, H. & GOODMAN, M. (1975) J.Biol.Chem. 250 : 4634

HAYES, J.D. (1976) B.Sc. Hons. dissertation, Univ. Edinburgh

HAYWARD, G.S. & SMITH, M.G. (1972) J.Mol.Biol. 63 : 383-396

HEDGPETH, J., GOODMAN, H.M. & BOYER, H.W. (1972) Proc.nat.Acad. Sci. (Wash.) 69 : 3448-3502

HEFFRON, F., BEDINGER, D., CHAMPOUX, J.J. & FALKOW, S. (1977) Proc.nat.Acad.Sci. (Wash.) 74 : 702-706

HELLING, R.B., GOODMAN, H.M. & BOYER, H.W. (1974) J.Virol. 14 : 1235-1244

HENDERSON, E., NAGANO, H., ZALKIN, H. & HWANG. L. (1970) J.Biol.Chem. 245 : 1416-1423

HENDERSON, D.H. & WEIL, J. (1975) Genetics 79 : 143-174

HERSCHFIELD, V., BOYER, H.W., YANOFSKY, C., LOVETT, M. & HELINSKI, D. (1974) Proc.nat.Acad.Sci. (Wash.) 71 : 3455-3459

HERSHEY, A.D. (ed.) 'The Bacteriophage Lambda' (1971), Cold Spring Harbor Lab., N.Y.

HERSKOWITZ, I. & SIGNER, E.R. (1970) J.Mol.Biol. 47 : 545-556

HOHN, B. (1975 a,b) J.Mol.Biol. 98 : 93-106 and 107-120

HOHN, B. & HOHN, T. (1974) Proc.nat.Acad.Sci. (Wash.) 71 : 2372-2376

HONIGMAN, A. (1976) Nature 262 : 112-116

HORIUCHI, T., KOGA, H., INOKUCHI, H. & TOMIZAWA, J. (1969) Molec.gen.Genet. 104 : 51

HOROWITZ, J.P., CHUA, J., CURBY, R.J., TOMSON, A.J., DA ROOGE, M.A. FISHER, B.E., MAURICIO, J. & KLUNDT, I. (1964) J.Med.Chem. 7 : 574-583

HRADECNA, Z. & SZYBALSKI, W. (1969) Virology 38 : 473-477

HUBACEK, T. & GLOVER, S.W. (1970) J.Mol.Biol. 50 : 111-127

HUGHES, S.G. (1977) Biochem.J. 163 : 503-509

IKEMURA, T. & DAHLBERG, J.E. (1973) J.Biol.Chem. 248 : 5024-5032

IMAMOTO, F., MORIKAWA, N. & SATO, K. (1965) J.Mol.Biol. 13 : 139-182

IPPEN, K., SHAPIRO, J.A. & BECKWITH, J.R. (1971) J.Bact. 108 : 5-9

ITO, J. & YANOFSKY, C. (1969) J.Bacteriol. 97 : 734-742

JACKSON, D.A., SYMONS, A.H. & BERG, P. (1972) Proc.nat.Acad.Sci. (Wash.) 69 : 2904-2909

JACKSON, E.N. & YANOFSKY, C. (1972a) J.Mol.Biol. 69 : 307-313

JACKSON, E.M. & YANOFSKY, C. (1972b) J.Mol.Biol. 71 : 149-161

JACKSON, E.N. & YANOFSKY, C. (1973) J.Mol.Biol. 76 : 89-101

JACOB, F. & WOLLMAN, E.L. (1954) Ann.Inst. Pasteur (Paris) 87 : 653

JEPPENSEN, P.G.N. (1974) Anal.Biochem. 58 : 195-207

KAISER, A.D. (1957) Virology 3 : 42-61

KAISER, A.D. & HOGNESS, D.S. (1960) J.Mol.Biol. 2 : 392-415

KAISER, A.D. & JACOB, F. (1957) Virology 4 : 509-521

KASAI, T. (1974) Nature (London) 249 : 523-527

KLOTZ, L.C. & ZIMM, B.H. (1972) J.Mol.Biol. 77 : 519-529

KOGA, H., MIYAUCHI, T. & HORIUCHI, T. (1970) Molec.gen.Genet. 106 : 114

KORN, L.J. & YANOFSKY, C. (1976a) J.Mol.Biol. 103 : 395-410

KORN, L.J. & YANOFSKY, C. (1976b) J.Mol.Biol. 106 : 231-242

LAM, S.T., STAHL, M.M., McMILIN, K.D. & STAHL, F.W. (1974) Genetics 77 : 425-433

LARGIN , M. & BELSER, W. (1973) Genetics 75 : 19-22

LEBOWITZ, P., WEISSMAN, S.M. & RADDING, C.M. (1971) J.Biol.Chem. 246 : 5120-5139

LEE, F., SQUIRES, C.L., SQUIRES, C. & YANOFSKY, C. (1976) J.Mol.Biol. 106 : 383-394

LEDERBERG, E.M. & COHEN, S.M. (1974) J.Bact. 119 : 1072-1074

LENNOX, E.S. (1955) Virology 1 : 190-206

LI, S. & YANOFSKY, C. (1972) J.Biol.Chem. 247 : 1031-1037

LIEB, M. (1970) J.Virol. 6 : 218

LIEDKE-KULKE, M. & KAISER, A.D. (1967) Virology 32 : 457-481

LINDAHL, G., SIRON, G., BIALLY, H. & CALENDAR, R. (1970) Proc.Nat.Acad.Sci. (Wash.) 66 : 587

MAIZELS, N.M. (1973) Proc.nat.Acad.Sci. (Wash.) 70 : 3585-3589

MANIATIS, T., PTASHNE, M., BARRELL, B.G. & DONELSON, J. (1974) Nature 250 : 394-397

MANIATIS, T., JEFFREY, A. & KLEID, D.G. (1975a) Proc.nat.Acad.Sci. (Wash.) 72 : 1184-1188

MANIATIS, T., PTASHNE, M., BACKMAN, K., KLEID, D.G., FLASHMAN, S., JEFFREY, A. & MAURER, R. (1975b) Cell 5 : 109-113

MALONE, R. & CHATTORAJ, D.K. (1975) Molec.gen.Genet. 143 : 35-41

MANDEL, M. & HIGA, A. (1970) J.Mol.Biol. 53 : 159-162

MANLY, K.F., SIGNER, E.R. & RADDING, C.M. (1969) Virology 37 : 177-188

MARGOLIN, P. (1974) In 'Metabolic Regulation' (ed.) GREENBERG, 12 : 389-446

MARMUR, J. (1961) J.Mol.Biol. 3 : 208-218

MATSUBARA, K. (1976) J.Mol.Biol. 102 : 427-439

MATSUSHIRO, A. (1963) Virology 19 : 475-482

MAURER, R., MANIATIS, T. & PTASHNE, M. (1974) Nature 249 : 221-223

MAYER, E.P., SMITH, O.H., FREDRICKS, W.W. & McKINNEY, M.A. (1975) Molec.gen.Genet. 137 : 131-142

McMILIN, K.D., STAHL, M.M. & STAHL, F.W. (1974) Genetics 77 : 409-423

MESELSON, M. (1964) J.Mol.Biol. 9 : 734-745

MILLER, R.C., BESMER, P., KHORANA, H.G., FIANDT, N. & SZYBALSKI, W. (1971) J.Mol.Biol. 56 : 363

MITCHELL, E., REZNIKOFF, W.G. & BECKWITH, J.R. (1970) J.Mol.Biol. 101 : 441-457

MORSE, D. (1972) J.Mol.Biol. 63 : 605-608

MOSTELLER, R.D. & YANOFSKY, C. (1970) J.Mol.Biol. 48 : 525-531

MUKAI, T., MATSUBARA, K. & TAKAGI, Y. (1976) Molec.gen.Genet. 146 : 269-274

MURIALDO, H. & SIMINOVITCH, L. (1972) Virology 48 : 785-823

MURRAY, N.E. & BRAMMAR, W.J. (1973) J.Mol.Biol. 77 : 615-624

MURRAY, N.E., BRAMMAR, W.J. & MURRAY, K. (1977) Molec.gen.Genet. 150 : 53-61

MURRAY, N.E., MANDUCA de RITIS, P. & FOSTER, L.A. (1973) Molec.gen.Genet. 120 : 261-281

MURRAY, N.E. & MURRAY, K. (1974) Nature (London) 251 : 476-481

NORKIN, L.C. (1970) J.Mol.Biol. 51 : 633

NOVICK, R.P. (1969) Bact.Rev. 33 : 210

OGAWA, T. & TOMIZAWA, J. (1968) J.Mol.Biol. 38 : 217-225

OLD, R., MURRAY, K. & ROIZES, G. (1975) J.Mol.Biol. 92 : 331-339

PARKINSON, J.S. & DAVIS, R.W. (1971) J.Mol.Biol. 56 : 425

PARKINSON, J.S. & HUSKEY, R. (1971) J.Mol.Biol. 56 : 369-384

PAULING, C. & HAMM, L. (1968) Proc.nat.Acad.Sci. (Wash.) 60 : 1495-1502

PHILIPPSEN, P. & DAVIS, R.W. (1976) Unpublished data

PIECZENIK, G., BARRELL, B.G. & GEFTER, M.L. (1972) Arch. Biochem. Biophys. 152 : 152-165

PIRROTTA, V. (1975) Nature 254 : 114-117

PIRROTTA, V. (1976) Curr.Topics in Microbiol. & immun. 74 : 21-54

PLATT, T., SQUIRES, C. & YANOFSKY, C. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 2399-2403

PLATT, T. & YANOFSKY, C. (1976) J.Mol.Biol. 103 : 411-420

POLISKY, B., BISHOP, R.J. & GELFAND, D.H. (1976) Proc.nat. Acad.Sci. (Wash.) 73 : 3900-3904

POUWELS, P.H. & PANNEKOEK, H. (1976) Molec.gen.Genet. 149 : 255-265

PRIBNOW, D. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 784-788

PTASHNE, M. (1974) Harvey Lectures 69 : 143

PTASHNE, M. & HOPKINS, N. (1968) Proc.nat.Acad.Sci. (Wash.) 60 : 1282

PTASHNE, M., BACKMAN, K., HUMAYUN, M.Z., JEFFREY, A., MAURER, R., MEYER, B. & SAUER, R.T. (1976) Science 194 : 156-161

RABBITTS, T. (1976) Nature 260 : 221-225

RAMAKRISHNAN, T. & ADELBERG, E.A. (1965) J.Bact. 89 : 661

RATNER, D. (1976) Nature 259 : 151-153

RATZKIN, B. & CARBON, J. (1977) Proc.nat.Acad.Sci. (Wash.) 74 : 487-491

REICHARDT, L.F. (1975) J.Mol.Biol. 93 : 267-288

ROBERTS, J.W. (1969) Nature 224 : 1168-1174

ROBERTS, J.W. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 3300

ROBERTS, J.W. & ROBERTS, C. (1975) Proc.nat.Acad.Sci. (Wash.) 72 : 147

ROBERTS, R.J. (1976) Crit.Rev.Biochem. 4 : 123-164

ROSE, J.K. & YANOFSKY C. (1974) Proc.nat.Acad.Sci. (Wash.) 71 : 3134-3138

RUGEON, F., KOURILSKY, P. & MACH, B. (1975) Nucl.Acids.Res. 2 : 2365-2378

SAKAKI, Y., KARU, A.E., LINN, S. & ECHOLS, H. (1973) Proc.nat. Acad.Sci. (Wash.) 70 : 2215-2219

SANGER, F. & COULSON, A.R. (1975) J.Mol.Biol. 94 : 441-448

SCHNÖS, M. & INMAN, R.B. (1970) J.Mol.Biol. 51 : 61-73

SGARAMELLA, V. (1972) Proc.nat.Acad.Sci. (Wash.) 72 : 3310-3314

SHARP, P.A., SUGDEN, B. & SAMBROOK, J. (1973) Biochemistry 12 : 3055-3063

SHINE, D. & DALGARNO, R. (1975) Nature 254 : 34

SHIMADA, K., WEISBERG, R.A., & GOTTESMAN, M.E. (1972) J.Mol.Biol. 63 : 483-503

SIGNER, E.R. (1969) Nature (London) 223 : 158-160

SIGNER, E.R. (1971) In 'The Bacteriophage Lambda' (ed.) HERSHEY, A.D. 139-174

SIMON, M.N., DAVIS, R.W. & DAVIDSON, N. (1971) In 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY : 313-328

SMITH, H.O. & NATHANS, D. (1973) J.Mol.Biol. 81 : 419-423

SO, M., GILL, F. & FALKOW, S. (1975) Molec.gen.Genet . 142 : 239-249

SOGO, J.M., GREENSTEIN, M. & SKALKA, A. (1976) J.Mol.Biol. 103 : 537-562

SQUIRES, C.L., LEE, F. & YANOFSKY, C. (1975) J.Mol.Biol. 92 : 93-111

STAHL, F.W., CRASEMAN, J.M. & STAHL, M.M. (1975) J.Mol.Biol. 94 : 203-212

STAHL, F.W., McMILIN, K.D., STAHL, M.M., MALONE, R., NOZU, Y. & RUSSO, V.E.A. (1972) J.Mol.Biol. 68 : 57-67

STAHL, F.W., McMILIN, K.D., STAHL, M.M., CRASEMAN, J.M. & LAM, S. (1974) Genetics 77 : 395-408

STAHL, F.W. & STAHL, M.M. (1975) Molec.gen.Genet. 140 : 29-37

STRUHL, K., CAMERON, J.R. & DAVIS, R.W. (1976) Proc.nat.Acad. Sci. (Wash.) 73 : 1471-1475

SUNSHINE, M., FEISS, M., STUART, J. & YOCHEM, J. (1977) Molec. gen.Genet. 151 : 27-34

SUSSMAN, R. & JACOB, F. (1962) C.R.Acad.Sci. (Paris) 254 : 1517

SZYBALSKI, E.H. & SZYBALSKI, W. (1974) Biochimie 56 : 1497-1503

TAKAHASHI, S. (1975) Molec.gen.Genet. 142 : 137-153

THOMAS, M. & DAVIS, R.W. (1975) J.Mol.Biol. 91 : 315-328

ULLMAN, A., JACOB, F. & MONOD, J. (1967) J.Mol.Biol. 24 : 339-343

UNGER, R.C. & CLARK, A.J. (1972 a,b) J.Mol.Biol. 70 : 531-537 and 539-548

VALENZUELA, E. (1976) J.Mol.Biol. 102 : 569-589

WANG, J.C. & KAISER, A.D. (1973) Nature New Biol. 241 : 16

WASMUTH, J.J. & UNBARGER, H.E. (1973) J.Bacteriol. 116 : 548-561

WEIL, J., CUNNINGHAM, R., MARTIN III, R., MITCHELL, E. & BOLLING, B. (1972) Virology 50 : 373-380

WEIL, J. & SIGNER, E.R. (1968) J.Mol.Biol. 34 : 273-279

WOOD, W.B. (1966) J.Mol.Biol. 16 : 118-133

YANOFSKY, C., CARLTON, B.C., GUEST, J.R., HELINSKI, D.R. & HENNING, U. (1964) Proc.nat.Acad.Sci. (Wash.) 51 : 266

YANOFSKY, C., DRAPEAU, G.R., GUEST, J.R. & CARLTON, B.C. (1967) Proc.nat.Acad.Sci. (Wash.) 57 : 296-298

YANOFSKY, C., HORN, V., BONNER, M., STASIOWSKI, S. (1971) Genetics 69 : 409-433

YANOFSKY, C. & ITO, J. (1966) J.Mol.Biol. 21 : 313-334

YOSHIMORI, R. (1971) Ph.D. Thesis, Univ. California, San Francisco

YOUNG, E.T., & SINSHEIMER, R.L. (1964) J.Mol.Biol. 10 : 562-564

YOUNG, E.T., & SINSHEIMER, R.L. (1968) J.Mol.Biol. 33 : 49-59

ZEIGER, R.S., SALOMON, R., DINGMAN, W. & PEACOCK, A.R. (1972) Nature, New Biology 238 : 65-69

ZISSLER, J., SIGNER, E.R. & SCHAEFER, F. (1971 a,b) in 'The Bacteriophage Lambda' (ed.) A.D. HERSHEY : 455-468 and 469-475